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**IMMUNOLOGICAL ANALYSIS OF HUMAN CHROMOSOMAL PROTEINS**

**Asaad A.M. Shallal**

**Thesis submitted to the University of Glasgow  
for the degree of Doctor of Philosophy**

**Department of Biochemistry**

**July, 1984**

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**To my Parents**

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## Abbreviations

The abbreviations used in this thesis are those suggested in the Instructions to Authors of the Biochemical Journal (1983) with the following additions:

A	Absorption
Ab	Antibody
Ag	Antigen
BSA	Bovine Serum Albumin
C'	Complement
C'F	Complement Fixation
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
GMEM	Glasgow Modification of Minimal Essential Medium
GS	Goat Serum
HAT	Hypoxanthine, aminopterin, thymidine
HT	Hypoxanthine, thymidine
Ig	Immunoglobulin
K	Kilo ( 1000)
MCA	Monoclonal Antibody
NEAA	Non-Essential Amino Acids
NHCP	Non-Histone Chromosomal Protein
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
PPO	2,5-diphenyloxazole

SDS	Sodium dodecyl sulfate
SLE	Systemic Lupus Erythromatosus
SRBCs	Sheep Red Blood Cells
TN	Tris-Saline
Tris	Tris(hydroxymethyl) methylamine
Triton-X100	Iso-octyl phenoxy polyethoxy ethanol
Tween 20	Polyoxyethylene (20) sorbiton monolaurate

## LIST OF CONTENTS

<u>Title</u>	<u>Page</u>
Title page	i
Acknowledgements	ii
Abbreviations	iii
Contents	v
List of Figures	xiii
List of Tables	xvii
Summary	xviii
<u>Chapter 1</u>	<u>Introduction</u>
	1
<u>1.1</u>	<u>Chromatin and Chromosome Structure</u>
	1
<u>1.1.1</u>	Nucleosome Structure
	1
1.1.1.1	Nucleosome Core Particle
	1
1.1.1.2	Histone H <sub>1</sub> -Containing Nucleosome
	1
1.1.1.3	The Linker DNA and Periodicity
	2
<u>1.1.2</u>	Supranucleosomal Structure
	3
<u>1.1.3</u>	Chromosome Structure
	4
1.1.3.1	Metaphase Chromosome
	4
1.1.3.2	Nucleoids
	6
<u>1.1.4</u>	Nuclear Pores
	6
<u>1.2</u>	<u>Control of Gene Expression</u>
	8
<u>1.2.1</u>	Control of Initiation of RNA Synthesis
	8
1.2.1.1	Modulator and Enhancer
	9
1.2.1.2	Initiators and Selectors
	9
<u>1.2.2</u>	Control at the Level of RNA
	Post-transcriptional Modifications
	11
1.2.2.1	Polyadenylation
	11
1.2.2.2	Capping
	12

<u>1.2.3</u>	Control by Differential Gene Splicing	13
1.2.3.1	The Viral System	14
1.2.3.2	The Immunoglobulin System	16
<u>1.2.4</u>	Translational Control	19
<u>1.2.5</u>	Control at the Level of DNA Methylation	21
<u>1.2.6</u>	Control at the Level of Chromatin Structure	23
1.2.6.1	Nuclease Sensitivity	23
1.2.6.2	Histone H <sub>1</sub>	25
1.2.6.3	High Mobility Group (HMG) Proteins	26
<u>1.3</u>	<u>Nonhistone Chromosomal Proteins</u>	28
<u>1.3.1</u>	Enzymes and Co-factors	29
<u>1.3.2</u>	Structural Proteins	31
<u>1.3.3</u>	Tissue and Cell Specific Proteins	33
<u>1.4</u>	<u>Characterisation of Nuclear and Nucleolar</u>	
	<u>Components by Immunological Approaches</u>	34
<u>1.4.1</u>	Antibodies to Nucleic Acids	34
<u>1.4.2</u>	Antibodies to Histones	38
<u>1.4.3</u>	Antibodies to Nonhistone Chromosomal Proteins	39
<u>1.4.4</u>	Antibodies to Nucleolar Antigens	42
<u>1.5</u>	<u>Monoclonal Antibodies</u>	43 X
<u>1.5.1</u>	History and Background	43
<u>1.5.2</u>	Properties and Limitations of Monoclonal Antibodies	47
<u>1.5.3</u>	Monoclonal Antibodies to Chromosomal Proteins	49
	<u>Concluding Remarks and Aim of Project</u>	53



<u>Chapter 2</u>	<u>Materials and Standard Solutions</u>	55
<u>2.1</u>	<u>Materials</u>	55
<u>2.1.1</u>	Cell Culture Materials	55
<u>2.1.2</u>	Disposable Plasticware	55
<u>2.1.3</u>	Radiochemicals	55
<u>2.1.4</u>	X-ray and Photographic Materials	56
<u>2.1.5</u>	Enzymes	56
<u>2.1.6</u>	Chemicals	56
<u>2.1.7</u>	Cell Lines	57
2.1.7.1	Human Cell Lines	57
2.1.7.2	Animal Cell Lines	57
<u>2.1.8</u>	Animals	57
2.1.8.1	Mice	57
2.1.8.2	Rabbits	57
<u>2.1.9</u>	Serological Materials	57
<u>2.1.10</u>	Sheep Red Blood Cells	60
<u>2.1.11</u>	Miscellaneous	60
<u>2.2</u>	<u>Standard Solutions and Buffers</u>	60
<u>2.2.1</u>	Fusion and Cell Culture Media	60
2.2.1.1	RPMI-1640 medium	60
2.2.1.2	Heat-Inactivation of FCS	61
2.2.1.3	HT and HAT media	61
2.2.1.4	GMEM medium	61
2.2.1.5	Polyethylene glycol (PEG) Solution	64
2.2.1.6	Antibiotics	64
2.2.1.7	Trypan Blue Dye Solution	64

<u>2.2.2</u>	Chromatin and Dehistonised Chromatin	
	Buffers and Solutions	64
2.2.2.1	Soluiton (1)	64
2.2.2.2	Solution (2)	64
2.2.2.3	Solution (3)	65
2.2.2.4	Solution (4)	65
2.2.2.5	2M Salt buffer	65
2.2.2.6	Sucrose buffer	65
2.2.2.7	SSC buffer	65
<u>2.3</u>	<u>Immunoassay Buffers</u>	65
<u>2.3.1</u>	ELISA buffer	65
2.3.1.1	Carbonate/EDTA Buffer (Antigen Coating Buffer)	65
2.3.1.2	Citrate-Phosphate Buffer	66
<u>2.3.2</u>	Complement Fixation Assay Buffers	66
2.3.2.1	Stock Diluent	66
2.3.2.2	Working diluent (W.D.)	66
<u>2.3.3</u>	Saline Buffers	66
2.3.3.1	Tris-Saline (TN)	66
2.3.3.2	Phosphate buffered Saline (PBS)	66
<u>Chapter 3</u>	<u>Methods</u>	67
<u>3.1</u>	<u>Preparation of Nuclei and Chromatin</u>	67
<u>3.1.1</u>	Preparation of Nuclei	67
3.1.1.1	The Method of Chauveau <u>et al</u>	67
3.1.1.2	Polyamine Method	67
<u>3.1.2</u>	Chromatin Preparation	68
<u>3.1.3</u>	Dehistonised Chromatin	69
3.1.3.1	2M Salt-extracted Chromatin	69
3.1.3.2	2M Salt-5M urea extracted chromatin	69



<u>3.1.4</u>	Bhorjee and Pederson's Method of Preparation of Nucleoli and Nonhistone Chromosomal Proteins	70
<u>3.2</u>	<u>Animal Procedures</u>	71
<u>3.2.1</u>	Preparation of immunogen in complete Freund's Adjuvent (CFA)	71
<u>3.2.2</u>	Injection of Mice	71
<u>3.2.3</u>	Injection Protocol of Rabbits	71
<u>3.2.4</u>	Fluid Withdrawal	71
3.2.4.1	From Mice	71
3.2.4.1a	Blood Withdrawal	71
3.2.4.1b	Ascitic Fluid Withdrawal	71
3.2.4.2	From Rabbits	72
<u>3.3</u>	<u>Lymphocyte Hybridoma Production and Tissue Culture</u>	72
<u>3.3.1</u>	Prerparation of Myeloma Cells	72
<u>3.3.2</u>	Preparation of Spleen Cells	72
<u>3.3.3</u>	Assessment of Cell Viability and Counting	72
<u>3.3.4</u>	Feeder Layer	73
<u>3.3.5</u>	Fusion Protocol	73
<u>3.3.6</u>	Cloning by Limited Dilution	74
<u>3.3.7</u>	Freezing of Cells	74
<u>3.3.8</u>	Thawing of Cells	74
<u>3.3.9</u>	Propagation of Monoclonal Antibodies	75
3.3.9.1	<u>In vivo</u>	75
3.3.9.2	<u>In vitro</u>	75
3.3.1.10	Routine Culture	75

<u>3.4</u>	<u>Assays of Monoclonal Antibodies and Other Antisera</u>	75
<u>3.4.1</u>	Radioimmunoassay	75
<u>3.4.2</u>	Enzyme Linked Immunosorbent Assay (ELISA)	76
<u>3.4.3</u>	Complement Fixation Assay (C'F)	77
3.4.3.1	Preparation of Complement (C')	77
3.4.3.2	Preparation of Antibody-Antigen-Complement Mixture (Ab-Ag-C')	77
3.4.3.3	Activation of Sheep Red Blood Cells (SRBCs)	77
3.4.3.4	Determination of the Percentage of Complement Fixed by the Antibody-Antigen Complex	78
<u>3.4.4</u>	Determination of the pH Effect on Monoclonal Antibody in ELISA	78
<u>3.4.5</u>	Competition Assay	79
<u>3.4.6</u>	Enzyme Treatment of HeLa Chromatin Reacting with Monoclonal Antibody	79
<u>3.5</u>	<u>Immunological and Immunochemical Methods</u>	80
<u>3.5.1</u>	Chemical Fractionation of Antibodies	80
<u>3.5.2</u>	Analysis of Biosynthetically Radiolabelled Immunoglobulin	81
3.5.2.1	Radiolabelling	81
3.5.2.2	Cell Lysis and Immunoprecipitation	81
3.5.2.2a	Cell Lysis	81
3.5.2.2b	Immunoprecipitation	82
3.5.2.3	Polyacrylamide Gel Electrophoresis Analysis	82
3.5.2.4	Fluorography	82
<u>3.5.3</u>	Antibody Radiolabelling	83
<u>3.5.4</u>	Immunoblotting	85
3.5.4.1	ELISA Technique	85
3.5.4.2	Immunoblotting by Radiolabelled Antibody	85
3.5.4.3	Immunoblotting by <sup>125</sup> I-Protein A Method	85

<u>3.6</u>	<u>Gel Electrophoresis and Protein Blotting</u>	86
<u>3.6.1</u>	Slab Gel Electrophoresis	86
<u>3.6.2</u>	Staining and Destaining of Gels	88
<u>3.6.3</u>	Protein Blotting	88
3.6.3.1	Electroblotting	88
3.6.3.2	Blotting by Diffusion	89
3.6.3.3	Staining of Blots	89
3.6.3.4	Destaining of Blots	89
3.6.3.5	DNA Binding on the Blot	90
<u>3.7</u>	<u>Immunofluorescence</u>	90
<u>3.7.1</u>	Preparation of Monolayer Cells	90
<u>3.7.2</u>	Preparation of Mitotic Cells	90
<u>3.7.3</u>	Lysis and Fixation of Cells to Preserve Keratins	91
<u>3.7.4</u>	Cells Preparation on Coverslips for General Immunofluorescence Staining	91
<u>3.7.5</u>	Treatment of Cells with Antibodies	92
<u>3.7.6</u>	Chromosome Spread Preparation	92
<u>3.7.7</u>	Chromosome Staining	93
<u>3.7.8</u>	Photography	93
<u>3.8</u>	<u>Miscellaneous</u>	
<u>3.8.1</u>	DNA Preparation	93
<u>3.8.2</u>	DNA Digestion	94
<u>3.8.3</u>	DNA Reconstitution	94
<u>3.8.4</u>	DNA Estimation	95
<u>3.8.5</u>	Protein Estimation	95
<u>3.8.6</u>	Intermediate Filaments Preparation (I.F.)	97
<u>3.8.7</u>	Nuclear Lamina Protein - Matrix Preparation	97

<u>Chapter 4</u>	<u>Results and Discussion</u>	100
<u>4.1</u>	<u>Results Obtained Using R3.1 Antiserum</u>	101
	Results	101
	Discussion	124
<u>4.2</u>	<u>Results Obtained Using R2.1 Antiserum</u>	131
	Results	131
	Discussion	151
<u>4.3</u>	<u>Results and Discussion of Monoclonal HNo-G7</u>	158
	Results	158
	Discussion	184
<u>Chapter 5</u>	<u>General Discussion</u>	197
<u>References</u>		202

LIST OF FIGURES

<u>Number</u>	<u>Headings</u>	<u>Page</u>
1	<u>Diagrammatic representation of the processing of the transcript of a gene containing a single intron</u>	10
2	<u>Initiation of protein synthesis</u>	19
3	<u>Schematic representation of the selection of monoclonal antibody - producing hybrids</u>	45
4	<u>Elution profile of radiolabelled (<sup>125</sup>I) anti-rabbit (or anti-mouse) IgG</u>	84
5	<u>Standard Curve for DNA Estimation</u>	96
6	<u>Standard Curve for Protein Estimation</u>	98
4.1.1	<u>ELISA of R3.1 Antiserum with HeLa Chromatin</u>	102
4.1.2	<u>ELISA of R3.1 Antiserum with HeLa Digested- and Undigested-chromatins</u>	103
4.1.3	<u>C'F Assay of R3.1 Antiserum with HeLa Digested- and Undigested-chromatins</u>	104
4.1.4a	<u>Competition ELISA of HeLa Chromatins Reactions with R3.1 Antiserum</u>	106
4.1.4b	<u>Percentage of Competition ELISA of HeLa Chromatins Reaction with R3.1 Antiserum</u>	106
4.1.5	<u>Immunoblots of HeLa Subcellular Fractions Reacted with R3.1 and Normal Rabbit Antisera</u>	107
4.1.6	<u>FITC-staining of HeLa Cells Reacted with R3.1 Antiserum</u>	110
4.1.7	<u>FITC-staining of HeLa Nuclei Reacted with R3.1 Antiserum</u>	111



4.1.8	<u>FITC-staining of Human Cells Reacted with R3.1</u>	
	<u>Antiserum</u>	112
4.1.9	<u>FITC-staining of HeLa Cells Reacted with</u>	
	<u>Normal Rabbit Serum</u>	113
4.1.10	<u>ELISA of R3.1 Antiserum with HeLa Lamina</u>	
	<u>Preparation</u>	115
4.1.11a	<u>C'F Assay of R3.1 Antiserum with Human</u>	
	<u>Chromatins</u>	116
4.1.11b	<u>C'F Assay of R3.1 Antiserum with Non-human</u>	
	<u>Chromatins</u>	117
4.1.12a	<u>ELISA of R3.1 Antiserum with Human Chromatins</u>	118
4.1.12b	<u>ELISA of R3.1 Antiserum with Non-human Chromatins</u>	119
4.1.13	<u>FITC-staining of HeLa Mitotic Cells Reacted</u>	
	<u>with R3.1 Antiserum</u>	122
4.1.14	<u>FITC-staining of Mitotic HeLa Cells</u>	
	<u>Cytocentrifuged and Reacted with R3.1 Antiserum</u>	123
4.2.1a	<u>C'F Assay of R2.1 with Human Chromatins</u>	132
4.2.1b	<u>C'F Assay of R2.1 with Non-human Chromatins</u>	133
4.2.2a	<u>ELISA of R2.1 with Human Chromatins</u>	134
4.2.2b	<u>ELISA of R2.1 with Non-human Chromatins</u>	135
4.2.3	<u>ELISA of R2.1 with Nuclease-digested and</u>	
	<u>-undigested HeLa Chromatins</u>	137
4.2.4	<u>Protein Blot of HeLa Chromatin Reacted with</u>	
	<u>R2.1 and Normal Rabbit Sera</u>	138
4.2.5	<u>FITC-staining of HeLa Cells Reacted with R2.1</u>	140
4.2.6	<u>FITC-staining of HeLa Keratin Structures</u>	
	<u>Reacted with R2.1</u>	142
4.2.7	<u>Immunoblot of R2.1 Reacted with HeLa Subfractions</u>	
	<u>Compared with Human and Non-human Dehistonised</u>	
	<u>Chromatins</u>	143

4.2.8	<u>Autoradiography of R2.1 Reacted with HeLa</u>	
	<u>Subfractions</u>	145
4.2.9	<u>Immunoblot of R2.1 Reacted with HeLa</u>	
	<u>Subfractions</u>	147
4.2.10	<u>FITC-stained Mitotic HeLa Cells Reacted</u>	
	<u>with R2.1</u>	149
4.3.1a	<u>Fusion Schedule</u>	159
4.3.1b	<u>Hybridoma Colony of HNo-G7</u>	160
4.3.2	<u>Immunoglobulin Secretion of HNo-G7</u>	161
4.3.3	<u>pH Effect on HNo-G7 Immunoreactivity</u>	162
4.3.4	<u>Effect of Ag- Coating Buffer</u>	163
4.3.5a	<u>A Chequerboard ELISA of HNo-G7 with HeLa</u>	
	<u>Chromatin</u>	165
4.3.5b	<u>ELISA of HNo-G7 with HeLa Chromatin</u>	166
4.3.6	<u>ELISA of HNo-G7 with Human Chromatins</u>	167
4.3.7a	<u>Competition ELISA of HNo-G7 with Human</u>	
	<u>Chromatins</u>	169
4.3.7b	<u>Percentage of Absorbed HNo-G7 Used in Competition</u>	
	<u>ELISA of Human Chromatins</u>	170
4.3.8	<u>ELISA of HNo-G7 with HeLa Subfractions</u>	171
4.3.9a	<u>Competition ELISA of HNo-G7 with HeLa</u>	
	<u>Subfractions</u>	172
4.3.9b	<u>Percentage of Absorbed HNo-G7 Used in</u>	
	<u>Competition ELISA of HeLa Subfractions</u>	173
4.3.10	<u>ELISA of HNo-G7 with Enzymes-Treated HeLa</u>	
	<u>Chromatin</u>	175
4.3.11	<u>FITC-staining of HeLa Cells Reacted with</u>	
	<u>HNo-G7 and 653 Culture Supernatants</u>	176

4.3.12	<u>FITC-control of HeLa Cells Reacted with</u> <u>RPMI-FCS</u>	177
4.3.13	<u>Giemsa Staining of HeLa Metaphase Chromsomes</u>	179
4.3.14a	<u>Immunoblot of HNo-G7 Reacted with HeLa</u> <u>Dehistonised Chromatins</u>	180
4.3.14b	<u>Autoradiograph of HNo-G7 Reacted with</u> <u>Dehistonised Chromatins</u>	181
4.3.14c	<u>Autoradiograph of Control Immunoblot</u>	182
4.3.15a	<u>Protein Blot of Human Chromatins</u>	189
4.3.15b	<u>Protein Blot of HeLa Subfractions</u>	190
4.3.15c	<u>SDS-Gel of HeLa chromatin fractions</u>	190



LIST OF TABLES

<u>Number</u>	<u>Heading</u>	<u>Page</u>
1	<u>Enzyme Components of Nonhistone Chromosomal</u> <u>Proteins</u>	30
2a	<u>Human Cell Lines</u>	58
2b	<u>Non-human Cell Lines</u>	59
3	<u>Composition of RPMI 1640 Medium</u>	62
4	<u>Composition of Glasgow Modified Eagle's</u> <u>Medium (GMEM)</u>	63
5	<u>The Ratio of DNA:Protein of Chromatin from</u> <u>Human Cell Lines</u>	121
6	<u>Nucleolar Antigens</u>	195

## Summary

Chromosomal proteins are exceptionally difficult to analyse by biochemical techniques because of their complexity, heterogeneity and insolubility. An immunological approach is therefore of considerable value since it may be employed to identify antigens within the cell by immunocytochemistry, to test for the distribution of antigens among different cell phenotypes by ELISA and complement fixation and to characterise protein antigens by immunoblotting.

Immunological analysis of the sera from two rabbits and a single monoclonal antibody has been undertaken in detail. One serum, from rabbit 3.1, was obtained from a rabbit which was immunised with HeLa chromatin extracted with 2M NaCl and 5M urea. The antibodies showed a moderately strong preference for HeLa chromatin. The major antigens in this antiserum required DNA for immunological activity in complement fixation and ELISA. The molecular weights of the major antigens on immunoblotting in the absence of DNA were in the range 43,000-45,000. In the presence of DNA, faint additional bands at molecular weight 68,000-70,000 could be detected. The discrepancy between the three techniques suggests that the major antigens detected on these assays may differ. In particular, the immunoblotting technique may not detect complex interactions involving more than one protein antigen in combination with DNA. Immunocytochemical analysis suggested that the antigen localised in the nuclei and in particular, in the peripheral area generally associated with envelope, matrix or lamina proteins. Preparations of lamina proteins showed considerable reaction with the antiserum so that at least some of the major antigens are in this fraction. This does not preclude the presence of these or other major antigens in the nuclear matrix or envelope.

In rabbit 2.1, antiserum to chromatin extracted with only 2M NaCl was produced. This gave a totally different spectrum of antigens with many of the major antigens having cytoplasmic localisation. Antigens detected by immunoblotting included proteins present in 2M NaCl extracted chromatin and also present in keratin preparations. the reaction with HeLa chromatin was preferential but not specific and immunoblotting indicated that the major antigens detected by this technique were present in a large number of cell lines.

The difference in response between the two rabbits could be correlated with the fact that they were not syngeneic. However, it is more likely that the major antigens in rabbit 2.1 are extracted by the additional 5M urea used to prepare the immunising antigen in rabbit 3.1.

Many of the difficulties associated with the analysis of polyclonal serum samples raised to a heterogeneous antigen are removed by hybridoma technology. A murine monoclonal antibody, HNo-G7, detected a single specific antigen which was not apparent in the analysis of the serum of either rabbit. The mouse was immunised with 2M NaCl 5M urea extracted chromatin of the type used to immunise rabbit 3.1. This monoclonal antibody, localises strongly on the nucleoli of HeLa cells, has a preference for epithelial cells and HeLa cells in particular, and reacts with chromatin which has been treated with RNase A and DNase I. It also does not react with HeLa DNA alone. Sensitive immunoblotting showed a molecular weight of 45,000.

The data emphasise the difference between conventional serological analysis and monoclonal antibody technology. Among the most important of these is the fact that HNo-G7 is a fully stabilised monoclonal antibody which can be synthesised in culture indefinitely and produced from frozen hybridoma cells. It can, therefore, be produced in whatever quantities are required and be utilised in the future to further investigate the role of its antigen in processes involved in nucleolar function.

**Introduction**



## 1. INTRODUCTION

### 1.1 Chromatin and Chromosome Structure

#### 1.1.1 Nucleosome Structure

It is now widely accepted that the nucleosome is the elementary repeat subunit of chromatin structure in eukaryotes. The nucleosome consists of an internal core of histones surrounded by DNA (for review see Felsenfeld, 1978; McGhee & Felsenfeld, 1980; Laskey, 1981; Igo-Kemenes et al, 1982). The globular core particle is sealed off by another species of histone,  $H_1$ , which is usually attached to the complex at the entry and exit of DNA (Thoma et al, 1979).

##### 1.1.1.1 Nucleosome Core Particle

The first level of chromatin structure organisation is the core particle. It has flat cylindrical shape with a diameter of 11.0nm and a height of 55 nm (Finch et al, 1977). The core consists of an octamer of the basic histone proteins, two molecules of each  $H_2A$ ,  $H_2B$ ,  $H_3$  and  $H_4$ . This octameric particle is surrounded by 146 base pair (bp) of DNA (Lutter, 1979) forming 1.75 turns. This is further extended by another 10 bp at each end to form a supercoil of a full two-turns comprising 166.0 bp surrounding the particle (Thoma et al, 1979). The 166 bp particle has been termed "Chromatosome" (Simpson, 1978). The nucleosome structure and its involvement in gene transcription and initiation has been the subject of intense research interest over the last few years (Lohr, 1981, 1983; Weisbrod, 1982; Paul, 1982; Oda, 1983).

##### 1.1.1.2 Histone $H_1$ -Containing Nucleosome

The next level in the organisation of chromatin structure is the "Mononucleosome". The mononucleosome consists of the core particle, the linker DNA and one molecule of the lysine-rich histone protein ( $H_1$ ). The involvement of  $H_1$  in the organisation of the nucleosome has been the focus of many research groups (reviewed by McGhee & Felsenfeld, 1980).

From nuclease digestion experiments, it has become clear that the globular core particle is well protected.  $H_1$  histone is located at the entry and exit of the 166.0 bp superhelical DNA (Simpson, 1978; Moyne et al, 1981; Thoma et al, 1979). It has been shown that  $H_1$  histone species consists of three distinct domains. The central one is a globular protein subunit (Isenberg, 1979) which is in close contact with the histone octamer and is able to close two full turns of DNA around the core (Allan et al, 1980).

#### 1.1.1.3 The Linker DNA and Periodicity

The chromosomes are linked with each other by "Linker" or "Spacer" DNA. The linker length varies from zero bp in lower eukaryotes (Noll, 1976; Morris, 1976; Thomas & Furber, 1976) to around 80 bp in sea urchin sperm (Spadafora et al, 1976, 1978). A considerable amount of attention has been paid to the organisation of internucleosomal DNA (Lawrence & Goeltz, 1981; Strauss & Prunell, 1982, 1983; Karpov et al, 1982). Data from nuclease digestion experiments have suggested that there may be classes of different spacer lengths in different organisms and tissues from the same organism (Kornberg, 1977; Chambon, 1978). This has been substantiated using different kinds of nucleases as well as a combination of nucleases. Using micrococcal nuclease, Karpov et al (1982) have reported the presence of 20, 30 and 40 bp spacers in Drosophila. They have also concluded that  $10n$  bp ( $n=2,3$  and 4) long intercore DNA is organised by  $H_3$  histone in particular and together with the core DNA forms a continuous superhelix. In rat liver chromatin, the highly variable lengths of linker DNA within the same cell, have been studied extensively by Prunell and Kornberg. They have developed a direct method, using a combination of nucleases, by which nucleosome dimers are trimmed to 166 bp of the core position retaining  $H_1$  histone. In rat liver chromatin, the mean value of the centre-to-centre distance between nucleosomes has been found to be



190 bp by using exonuclease III (Prunell & Kornberg, 1982; Strauss & Prunell, 1982). A considerable difference of the helical periodicity of DNA on the nucleosome is found in the literature, due to variable interpretations of the nuclease digestion data. By electrophoresis of nuclease digests in denaturing gels, DNA fragments give rise to a characteristic ladder pattern with fragments spaced about 10.0 nucleotides apart (Noll, 1974). However, Lutter (1979) and Bryan et al (1979) have noted a 10.4 nucleotide spacing in DNase-I digestion. Simpson and Stafford (1983) have shown recently, by using cloned segments of DNA in vitro, that a cutting site spacing of near 10.0 nucleotides occurs for a large part of a reconstituted nucleosome. This reflects a DNA helical repeat of 10.0 bp in the core particle. Klug and Lutter (1981) have concluded that the variation of angle of attack of nucleases deduced on the basis of a 10.5 fold screw in the DNA double helix leads to steric inaccessibilities, whereas all data are compatible with periodicity of 10.0 bp per turn of the helix. An earlier conclusion by Finch et al (1977) in what they described as the "Linking-number paradox" has suggested that in solution, the helical twist of free DNA corresponds to a full turn per 10.5 bp. When coiled around the histone core, the helical twist of the DNA corresponds to a full turn per 10.0 bp.

#### 1.1.2 Supranucleosomal Structure

It is now well accepted that the first level of DNA condensation is provided by nucleosome structure which in low salt concentration gives rise to a (nucleosomal) beaded chain (Oudet et al, 1975; McGhee et al, 1980; Thoma & Koller, 1977; Thoma et al, 1979). At the second level of chromatin organisation, the beaded chain (nucleofilament) condenses in solution, containing magnesium or a high concentration of monovalent cations, to form a thick supercoil or "Solenoidal Structure" of a pitch around 11nm (Finch & Klug, 1976). The thick fibre diameter in high salt concentration has been reported to be around 25-30nm in nuclease

digestion experiments (Finch & Klug, 1976), by electron microscopy (Thoma et al, 1979) and by electric dichroism (McGhee et al, 1980).

Moreover, a radial arrangement for the faces of chromatosomes around the fibre axis of 30nm solenoid has been suggested (Finch & Klug, 1976; McGhee et al, 1980). In this model, six chromatosomes, each of 11nm in diameter and 5.5nm thick, are arranged per turn of left handed solenoid. Evidence for the 6-7 nucleosomes per turn in the solenoid fibre has been substantiated by different techniques such as electron microscopy, neutron diffraction and x-ray scattering (for review see Igo-Kemenes et al, 1982).

An alternative model to the solenoid is the "Superbead". Renz et al (1977), using electron microscopy, have reported the presence, at high ionic strength, of a fibre of 20 nm in diameter, composed of discrete globular structures that are held by H<sub>1</sub> histone. This superbead structure has been supported by further work on hen erythrocyte chromatin. Chromatin fibre fragments with a length of 500 nm and an average diameter of 32 nm have been observed by electron microscopy. Each fibre has been found to consist of 13 globular subunits comprising a "Superbead". The globular subunits contain a different number of nucleosomes at an average of 17 (Meyer & Renz, 1979). Chromatin fibres of 33 nm long and 30 nm in diameter have been also reported in a variety of different systems (Subirana et al, 1981). Biochemical studies supporting the superbead model suffer from the lack of repeated periodicity of DNA pattern by nuclease digestion (Igo-Kemenes et al, 1982).

### 1.1.3 Chromosome Structure

#### 1.1.3.1 Metaphase Chromosome

There is accumulating evidence that the 25 nm chromatin fibre appears to be folded further both in interphase nuclei (Benyajati & Worcel, 1976; Igo-Kemenes & Zachau, 1978; Adolph, 1980a; Lebkowski & Laemmli, 1982), and in metaphase chromosomes (Adolph et al, 1977a; Lewis



& Laemmli, 1982; Earnshaw & Laemmli, 1983). The fibres in interphase and metaphase chromosomes have been found to be arranged in the form of loops or domains of 50 -100 Kb of DNA which are constrained by protein framework (Paulson & Laemmli, 1977; Igo-Kemenes & Zachau, 1978).

Stubblefield and Wray (1971) have proposed that mitotic chromosomes consist of distinct axial and peripheral chromatin components.

Recently, Paulson and Laemmli (1977) have shown that, in dehistonised chromosome preparations, loops of DNA are seen surrounding a residual axial structure.

A general radial loop model has been suggested in which chromatin loops emanate from a central axis formed by a network of nonhistone proteins designated as the "scaffolding" that links the bases of the loops (Laemmli et al, 1978). Support for such a model comes from thin-section electron microscopy of swollen metaphase chromosomes (Adolph, 1980b; Hozier et al, 1981) and swollen cells (Adolph, 1981).

The scaffold structure is composed primarily of two high molecular weight proteins (Sc1 and Sc2) whose interactions are believed to be stabilised by metallo-protein interactions (Lewis & Laemmli, 1982). Further support for the involvement of scaffold proteins, comes from nuclease digestion studies of chromosomes after low salt protein extraction which reveal a nonhistone protein residual scaffold, visible by electron microscopy (Earnshaw & Laemmli, 1983). These residual proteins have been found to be fibrous in nature, retaining the differentiated regions of the chromosomes derived from the "kinetochores" and chromatid axis (Earnshaw & Laemmli, 1983). It has also been noted that in the presence of magnesium or at high NaCl concentrations, the fibrous network undergoes a lateral aggregation which is reversible.

#### 1.1.3.2 Nucleoids

Structures resembling the nucleus and retaining its morphological features but depleted of protein have been known for many years (Cook & Brazell, 1975, 1980). These structures which have been called "Nucleoids" are released by gently lysing cells in solutions containing non-ionic detergent and high concentration of monovalent cations. The nucleoids consisted predominantly of superhelical, intact DNA, looped during lysis (Cook & Brazell, 1976; Lavin & Davidson, 1981). The linear nuclear and unbroken DNA has been found to be packaged within a cage of RNA and protein in these nucleoids. However, they lack any organised membrane structure (McCready et al, 1980).

A model based on the premise that replication takes place at the nuclear cage has also been suggested (McCready et al, 1980) and supported by the sequencing of loops of the nuclear DNA coding for the alpha- and beta-globin genes (Cook & Brazell, 1980). Recently, it has been proposed that regions of the chromosomes involved in replication may be preferentially bound to the nuclear matrix (Valenzuela et al, 1983). This idea has been supported by electron microscopy of DNA fragments showing an enrichment of replicating forks.

#### 1.1.4 Nuclear Pores

Electron microscopy of non-membranous ghosts from HeLa cell nuclei, has shown the presence of an array of "Rod-like" (250nm long and 60nm diameter) and "Annular" structures interconnected by thin strands (Keller & Riley, 1976). These strands are composed of double stranded DNA. The array is believed to be responsible for the spherical shape of the interphase nuclei. Candidates for the structural components that determine the nuclear shape have been isolated (Keller & Riley, 1976) and variously called the "Pore Complex-Lamina" (Aarson & Blobel, 1975) or "Nuclear Protein Matrix" (Berezney & Coffey, 1974). The major components of the ghost include annuli with inner and outer diameters

43nm and 90nm respectively which are consistent in dimensions with nuclear pore structures (Riley & Keller, 1978). It has also been proposed that the non-membranous layer could correspond to a structural nuclear framework or "Skeleton" which confines and possibly organises interphase nuclear chromatin and confers a degree of mechanical rigidity on isolated nuclei. These nucleoskeletons have been isolated from cells in all stages of the cell cycle except mitosis (Riley & Keller, 1978). Recently, it has been found that digestion of the thin connecting strands by DNases results in the destruction of the gross morphology of the nucleoskeleton and the release of thick rod- and pore-like structures (Detke & Keller, 1982). This has led to the conclusion that DNA is required to maintain the structural integrity of the nucleoskeleton. In addition, it has been shown that the major nucleoskeletal proteins are also present as major components of the chromosome scaffold. At least two of the nucleoskeletal proteins appear to be modified during the transition into mitosis suggesting that nuclear dissolution associated with open mitosis is accompanied by gross rearrangement of the nucleoskeletal elements to form components of metaphase chromosomes (Detke & Keller, 1982).



## 1.2 Control of Gene Expression

The control of gene expression has been a major focus of research over the past decade. The experimental results have provided the solution to a number of structural questions, but little has been achieved with respect to the actual mechanism. Many candidates have been suggested to play a part in this process including RNA, nonhistone chromosomal proteins and ribonucleoproteins. Research on the control of gene expression at the level of RNA processing and DNA modification has been particularly intensive and has shown considerable progress.

### 1.2.1 Control of Initiation of RNA Synthesis

Eukaryotic mRNAs are synthesised in the nucleus of a cell as a part of a long transcriptional precursor unit referred to as heterogeneous nuclear RNA (hnRNA).

The development of new techniques in DNA and RNA sequencing has led to the identification of sequences in and near many structural genes which have common features (reviewed by Paul, 1982). The isolation of RNA polymerases has helped in understanding part of the mechanism of transcription. In addition, other mechanisms involved in processing and maturation of the genetic message in nuclei, and its transportation to the cytoplasm has become clearer. It is now widely accepted that many eukaryotic gene sequences on the DNA are discontinuous. The primary RNA transcripts (hnRNAs) which are derived from these DNA segments contain coding sequences (exons) and non-coding sequences (introns or intervening sequences) (Abelson, 1979).

The formation of mature eukaryotic mRNA in nuclei involves a complex series of reactions, including capping, methylation, polyadenylation and splicing, before being exported to the cytoplasm. A possible control mechanism of expression has been suggested at each level (Shatkin, 1976; Banerjee, 1980; Breathnach & Chambon, 1981; Busch et al, 1982; Knowler, 1982).

A simple schematic diagram which represents some important events in mRNA maturation is shown in figure 1.

#### 1.2.1.1 Modulator and Enhancer

The fact that some sequences located upstream of the appropriate genes are associated with a certain level of control of gene expression has attracted much interest. Sequences comparable to the promoter in prokaryotes have been reported in the sea urchin H<sub>2</sub>A histone gene (Grosschedl & Birnstiel, 1980a) and in cloned mutants of SV40 (Benoist & Chambon, 1980, 1981).

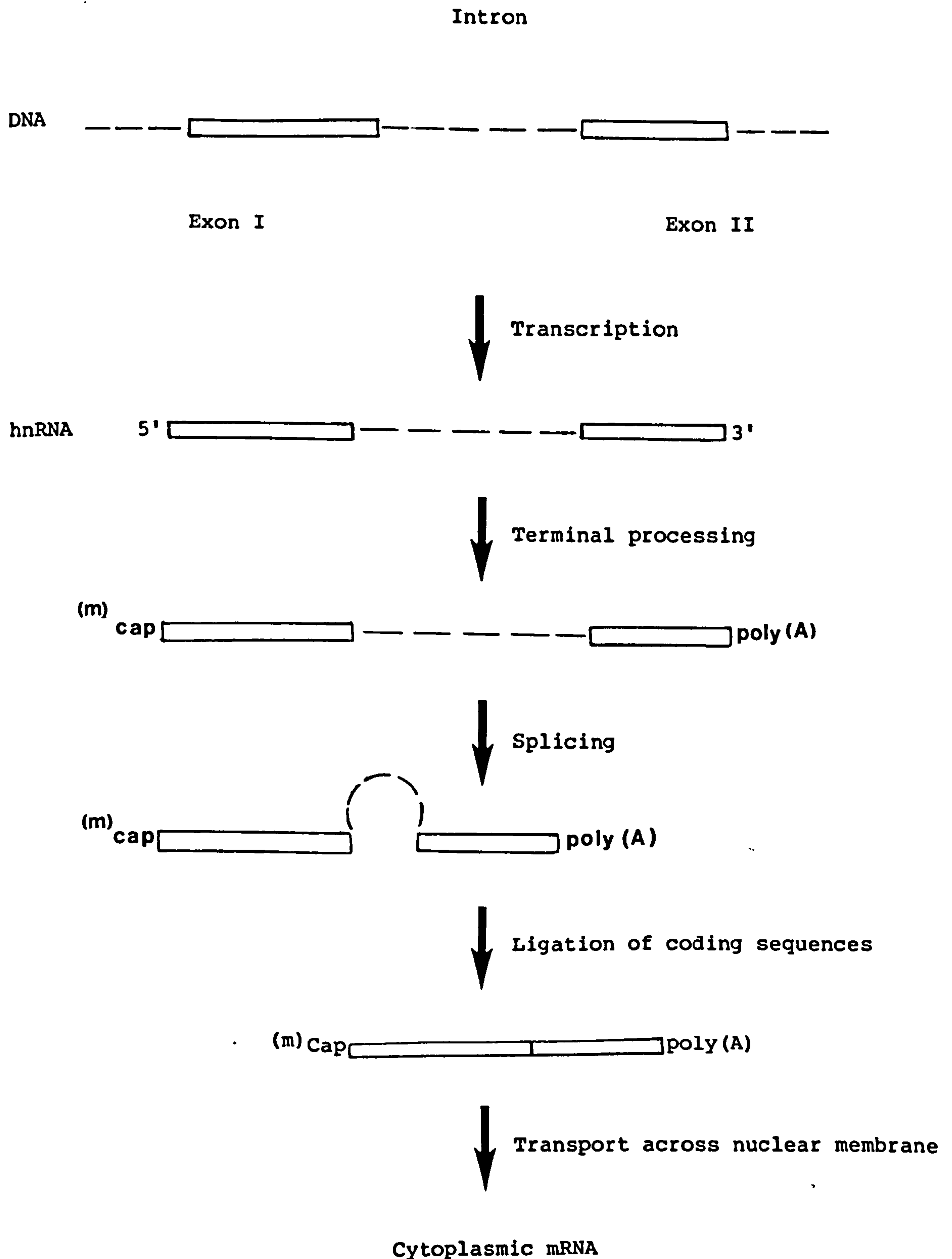
Recent interest has been focused on other sequences situated upstream of the initiation site of transcription. Grosschedl & Birnstiel (1980b) working on sea urchin H<sub>2</sub>A histone gene, have shown the presence of three functional DNA segments located upstream of the gene, given the names, "Modulator Selector & Initiator" elements. The modulator has been shown to control the rate at which the specificity elements operate. Dierks et al (1981) have reported a 10 bp sequence located before the cap site and also found preceding rabbit beta-globin genes and many other genes at a similar location. This sequence may be involved in modulating the efficiency of transcription.

Sequences called "Enhancers" with analogous characteristics to the modulator described earlier, have also been reported in the eukaryotic viral gene of SV40. Banerji et al (1981) have found a 72 base sequence which greatly enhances transcription of genes located upstream of the initiation site of the gene.

#### 1.2.1.2 Initiators and Selectors

Transcriptional initiation is one level of the control of eukaryotic gene expression. The specificity for the location and the frequency of transcription initiation resides in the upstream 5'-sequences for genes transcribed by RNA polymerase II (Breathnach & Chambon, 1981) and probably by RNA polymerase I (Grummt, 1981).

Figure 1 Diagrammatic representation of the processing of the transcript  
of a gene containing a single intron





Sequences situated upstream from the initiation site of specific genes have been reported to be responsible for transcription initiation. A possible consensus sequence for such initiation is called the "Chambon box" (Benoist et al, 1980; Struhl, 1981). This sequence resides approximately 80 bp upstream.

Another consensus sequence commonly centred about 25-30 bp upstream from the cap site has also been reported in most of the genes sequenced so far. This (AT) rich sequence which is known as "TATA" box was first noted by Goldberg and Hogness (see e.g. Breathnach & Chambon, 1981). It is believed that this sequence is necessary for accurate initiation, since in its absence multiple initiation points have been reported (Benoist & Chambon, 1981; Grosveld et al, 1982). It is also reported that upon deleting this sequence, a lower rate of initiation has been noted in other genes (Paul, 1982). Other workers, using mutants lacking the sequence upstream from position 29, have shown that specific transcription is not promoted in the absence of such consensus sequence (Corden et al, 1980). Recently, Davison et al (1983) have found that the TATA box region is involved in the stable binding of transcription factor(s).

In any case, since the TATA box is absent from some genes (Breathnach & Chambon, 1981), Grosschedl and Birnstiel (1980a, 1980b) have proposed that the TATA box acts as a "selector".

### 1.2.2 Control at the Level of RNA post-transcriptional Modifications

#### 1.2.2.1 Polyadenylation

Two important modifications commonly occur to hnRNA before it is processed further to yield mRNA. These modifications are polyadenylation at the 3'-end and capping at the 5'-end.

Evidence for the occurrence of poly(A) sequences in cytoplasmic mRNA was first reported by Edmonds and Caramela (1969). It has been shown that poly(A) is absent in nucleolar RNA (rRNA) but is present in the RNAs of both cytoplasm and nucleoplasm (Edmonds et al, 1971).

An important hexanucleotide sequence (AAUAAA) has been found to signal the post-transcriptional addition of polyadenylic acid. This hexanucleotide is located at about 25-30 nucleotides from the 3'-end of mRNA (Proudfoot & Brownlee, 1976), and it has been observed in all eukaryotic mRNAs studied so far. This sequence has also been found in the late gene of eukaryotic virus SV40 (Fitzgerald & Shenk, 1981). However, other components or structural features may also be involved. It is worth noting that in a number of genes, the hexanucleotide sequence is found in internal positions and does not induce polyadenylation. Moreover, several cases are known where transcription units contain more than one polyadenylation site (for review see Knowler, 1982).

The nature of the mRNA product from a transcript could be post-transcriptionally controlled by differential selection of polyadenylation sites (Nevins & Darnell, 1978). However, some evidence supports a role for the 3'-end poly(A) tail in extending the stability of mRNA and in the selection of the 3'-end mRNA chains during their biogenesis (Browerman, 1981). It has also been suggested that the polyadenylate may modulate mRNA transport across the nuclear membrane. However, histone mRNA which lacks a poly (A) tail, has been found to leave the nucleus much faster than the polyadenylated sequence (Andesnik & Darnell, 1972; Greenberg & Perry, 1972). Another possible role for the poly(A) sequence is its involvement in the splicing mechanism (Bina et al, 1981). This has been contradicted by Zeevi et al (1981) who have shown that splicing can occur in the absence of polyadenylation.

#### 1.2.2.2 Capping

5'-Terminal capping is one of the important post-transcriptional processes to which an hnRNA is subjected before maturation to yield mRNA. The unique 5'-cap was first shown by Reddy et al (1974). The 5'-end of the gene transcript is capped by the addition of a 7-methyl guanosine (Cap-0) through a pyrophosphate bond (Zimmern, 1975; Dubin &



Stollar, 1975). The cap may then be modified further by the 2'-O-methylation of the ribose moieties of the first or the first two nucleotides of the transcripts (Cap-1 and Cap-2 respectively) (Moyer & Banerjee, 1976; Rose, 1975; Sommer et al, 1976). The cap protects the mRNA against attack by phosphatases and other nucleases at its terminus. In addition, it promotes the mRNA's functional role at the level of initiation of translation (Rottman et al, 1974).

The 5'-termini of both mature mRNA and its precursors are identical and map at the same point on the gene sequence (Weaver & Weismann, 1979). This suggests that transcription is likely to be initiated at or very close to the 5'-end of the mRNA and little, if any, 5'-trimming occurs.

A number of eukaryotic genes have a tetranucleotide (CTCA) sequence immediately preceding the encoding sequence of mRNA which may represent a capping signal (Vankatesan et al, 1980).

Yamaguchi et al (1982) have observed another sequence which is complementary to the 3'-terminal of 18S rRNA, but situated in the 5'-noncoding region of eukaryotic mRNA. This sequence together with the 5'-cap structure enhance the rate of initiation of the complex formation in protein synthesis.

### 1.2.3 Control by Differential Gene Splicing

One of the major alterations which occur to the primary RNA transcript (hnRNA) is cutting various sequences (introns) and joining the remaining sequences (exons) in a process called "Splicing". This process produces a mature mRNA ready to cross the nuclear membrane to the cytoplasm. Splicing has attracted a considerable amount of attention in recent times as potential control mechanism (reviewed recently by Mount, 1982). The sites of splicing points at the exon-intron junctions has been the focus of interest. Mount (1982) has collected a catalogue containing the sequences of splicing sites from a large number of nuclear

as well as viral gene encoding proteins. These are called the consensus sequences. The catalogue has also confirmed the presence of GT/AG rule originally described by Breathnach et al (1978). The GT is present at the beginning of the intron and AG is at the end. This rule has been obeyed in the majority of the collected intron/exon sequences except for few cases like the  $\lambda$  immunoglobulin gene of MOPC-315 plasmacytoma (Bothwell et al, 1981) and in alpha-crystallin eye lens gene (King & Piatigorsky, 1983). However, splicing is not a simple mechanism and many nuclear proteins and poly-ribonucleotide (such as the small nuclear RNAs-snRNA) components are thought to be involved (Rogers & Wall, 1980; Lerner & Steitz, 1979; Lerner et al, 1980).

It is now well known that eukaryotic genes are subjected to various degrees of regulation at the transcriptional and post-transcriptional levels. Two categories of transcriptional units can be identified. A "simple" transcription unit which encodes single protein genes such as the histone genes (Hentschel & Birnstiel, 1981) and alpha- and beta-globin transcription units (Nishioka & Leder, 1979). On the other hand, the "complex" transcription units are those whose primary transcript can produce more than one mRNA encoding more than one protein. These transcripts contain two or more poly(A) sites, and two or more variations of post-transcriptional gene expression (Darnell, 1982). Two important systems of this type are the viral and immunoglobulin systems.

#### 1.2.3.1 The Viral System

Viral systems have been widely employed in the study of some of the mechanisms involved in gene structure and gene expression. Studies on the control of gene expression by differential transcript processing or splicing in viruses, have produced much information relating to the complex mechanism of mRNA maturation. This process has been extensively analysed in SV40 (Fiers et al, 1978; Shermann & Weisman, 1980), polyoma



(Soeda et al, 1980), adenovirus and other viruses (Darnell, 1982; Logan & Shenk, 1982) and more recently in influenza virus (Lamb & Lai, 1982).

In practical terms, RNA splicing is believed to be a control mechanism and/or a regulatory event. The adenovirus system provides a good model for this theory. The study of the formation of adenovirus mRNA has provided many of the details of eukaryotic mRNA biogenesis. Multiple steps are required to produce a functional mRNA including steps where alternative pathways are possible. In adenovirus, at early stages after infection, at least six transcription units are expressed. All of these units encode families of messenger ribonucleic acids whose members are differentially processed by splicing and as a result encode different polypeptides. At late (L) time after infection, new families of mRNAs are made and most of these are produced from a single transcription unit.

At early (E) time after infection, E1A gene region encodes two mRNAs which are produced by differential splicing. The E1A specific gene product facilitates expression of other mRNAs, E1B, E2, E3, E4 and L1 (Jones & Shenk, 1979). It is also likely that a variety of non-adenovirus genes are regulated by similar mechanisms. In addition, E1A not only acts at the level of initiation of transcription but may modulate certain cellular genes (Logan & Shenk, 1982).

Recently, analysis of the product of adenovirus L1 mRNA during early and late infection has provided evidence for the control of gene expression through an alternative splicing pathway (Chow et al, 1979). Evidence for the expression of a particular region of a genome which can be controlled through alternative splicing of the primary RNA transcripts has been found not only in viruses but also in cellular genes (Amara et al, 1982; King & Piatigorsky, 1983).

More recently, Mariman et al (1983), using mapping data from S1 nuclease digestion have shown that there are alternative splicing pathways for the excision of the first and second introns from RNAs that

are derived from the major late transcription unit in adenovirus. It is interesting to note that the first cleavages are introduced in the nascent RNA before the RNA polymerase has progressed more than 2000 nucleotides beyond the cleavage sites. It is also shown that intron (1) is preferentially removed before intron (2), however alternative pathways have also been found. Similarly, Sherman and Weissman (1980) have indicated that in SV40 virus, splicing is not strictly sequential.

Moreover, the expression of a transcription unit such as adenovirus unit, could also be controlled through differential poly(A) site selection at the post-transcriptional stem (Darnell, 1982). the same is true of the immunoglobulin heavy chain gene (see section 1.2.3.2). However, nuclear RNA splicing in adenovirus-2 mRNA formation has also been noted in the absence of poly(A) sites (Zeevi et al, 1981).

#### 1.2.3.2 The Immunoglobulin System

Antibodies are composed of two identical heavy- and <sup>two</sup>light-chain polypeptides. Each heavy (H) and light (L) chain contains an amino terminal, variable (V) region and a carboxyl end, constant (C) region. The variable regions are encoded by several hundred germ-line genes, while a very limited number of genes encode the (C) region. The various classes of immunoglobulins with different functions (IgM, IgD, IgG, IgE and IgA) are distinguished by different heavy chains defined by their constant regions. The mouse C<sub>H</sub> gene locus consists of eight genes ( $\mu$ ,  $\delta$ ,  $\gamma_3$ ,  $\gamma_{2a}$ ,  $\gamma_{2b}$ ,  $\gamma_1$ ,  $\epsilon$ ,  $\alpha$ ) (Marcu, 1982).

Vertebrate immune systems are able to respond specifically to an almost unlimited number of different foreign molecules (antigens). The production of immunoglobulins that bind specifically to the antigen comprises the major component of this response. The genes encoding immunoglobulins display several complex features associated with the need to generate such a large repertoire of specificities.



There are three unlinked immunoglobulin gene families: the light chain ( $\kappa$ ) and ( $\lambda$ ) gene families and a single gene family encodes heavy chains (Tonegawa, 1983). Variable regions show the most diversity; this is due to the role of the variable region to recognise vast varieties of immunogens (antigens). To create variable region diversity, at least one specific genetic mechanism exists; the process of DNA joining. Light chain variable regions are encoded by two gene segments, variable (V) and joining (J), which are separated in germ line DNA. The heavy chain variable regions are encoded by three such gene segments, (V), diversity (D) and (J) (Early et al, 1982, Marcu, 1982; Höcht1 & Zachau, 1983; Alt & Baltimore, 1982).

It has been found that during B-lymphocyte development and immunoglobulin synthesis, the ( $\mu$ ) heavy chain is first inserted as an integral protein in the plasma membrane. However, a similar ( $\mu$ ) chain has been detected as part of secreted immunoglobulins (Alt et al, 1980). It has also been observed that the mRNA isolated from B-lymphocyte tumour cell lines directs the synthesis of two forms of heavy chains, 67,000 and 64,000 in molecular weight. Rogers et al (1980) using cDNA clones of mouse B-lymphocyte tumours have noted the presence of two mRNAs which differ in their 3'-termini. One 3'-terminus has been found to dictate amino acid sequences appropriate for membrane-binding and the other is involved in secretion. This work has been substantiated by further analysis of the data obtained from the cloning experiments. Early et al (1980) in their analysis of the genomic cloned DNA data have revealed the presence of two poly(A) sites in the same transcription unit and the primary transcript. It has also been shown that depending on the choice of poly(A) site, two heavy chain mRNAs can be formed. These two mRNAs produce two different products; the  $\mu$ membrane (m) chain and the  $\mu$  secreted (S) one. Both  $\mu$ m and  $\mu$ s mRNAs are produced from transcripts of a single gene. The mapping

analysis data have also suggested that developmental control of the site, at which poly(A) is added to the transcripts of the  $\mu$  gene, determines the relative levels of  $\mu$ m or  $\mu$ s chain synthesis. Meanwhile, Singer et al (1980) working on human lymphoma cells have arrived at a similar finding regarding the presence of two kinds of mRNA coding for receptor and secretory forms of IgM  $\mu$ -chains. They have proposed that the synthetic pathways for these immunoglobulins diverge at the post transcriptional level, possibly by differential RNA splicing.

Moreover, murine cell lines producing IgG have also been found to produce two mRNA species for immunoglobulin  $\gamma$ -chains (Rogers et al, 1981). Membrane-bound or secreted antibody is thought to be expressed first by regulation of the site of transcriptional termination or poly(A) addition and then by site specific RNA splicing (Rogers et al, 1981).

Recently, Knapp et al (1982) have noted a simultaneous expression of immunoglobulin  $\mu$ - and  $\delta$ -chains from a cloned B-cell lymphoma, where a single copy of  $V_H$  gene is shared by two adjacent  $C_\mu$  and  $C_\delta$  genes. They argue that dual expression of  $\mu$ - and  $\delta$ -heavy chains using a single  $V_H$  gene is accomplished by alternate RNA splicing mechanism. Similar suggestion by Moore et al (1981) have also been reported earlier, using clones from mouse sperm DNA. They have also compared their results with that of rat myeloma DNA whose  $C_\mu$  gene has been deleted. They have concluded that two alternative mechanisms may be used in the expression of IgD molecules; RNA splicing in B-cells and DNA rearrangement in plasma cells. Other workers have also reported RNA splicing as a control mechanism (Maki et al, 1981) in immunoglobulin synthesis. They have also suggested that the same five exonic regions common to both  $\mu$  chains also appear in  $\delta$  chains.

The molecular basis of the presumed differential choices of poly(A) and splice sites is not fully known and it remains possible that other components or template changes may dictate a post-transcriptional



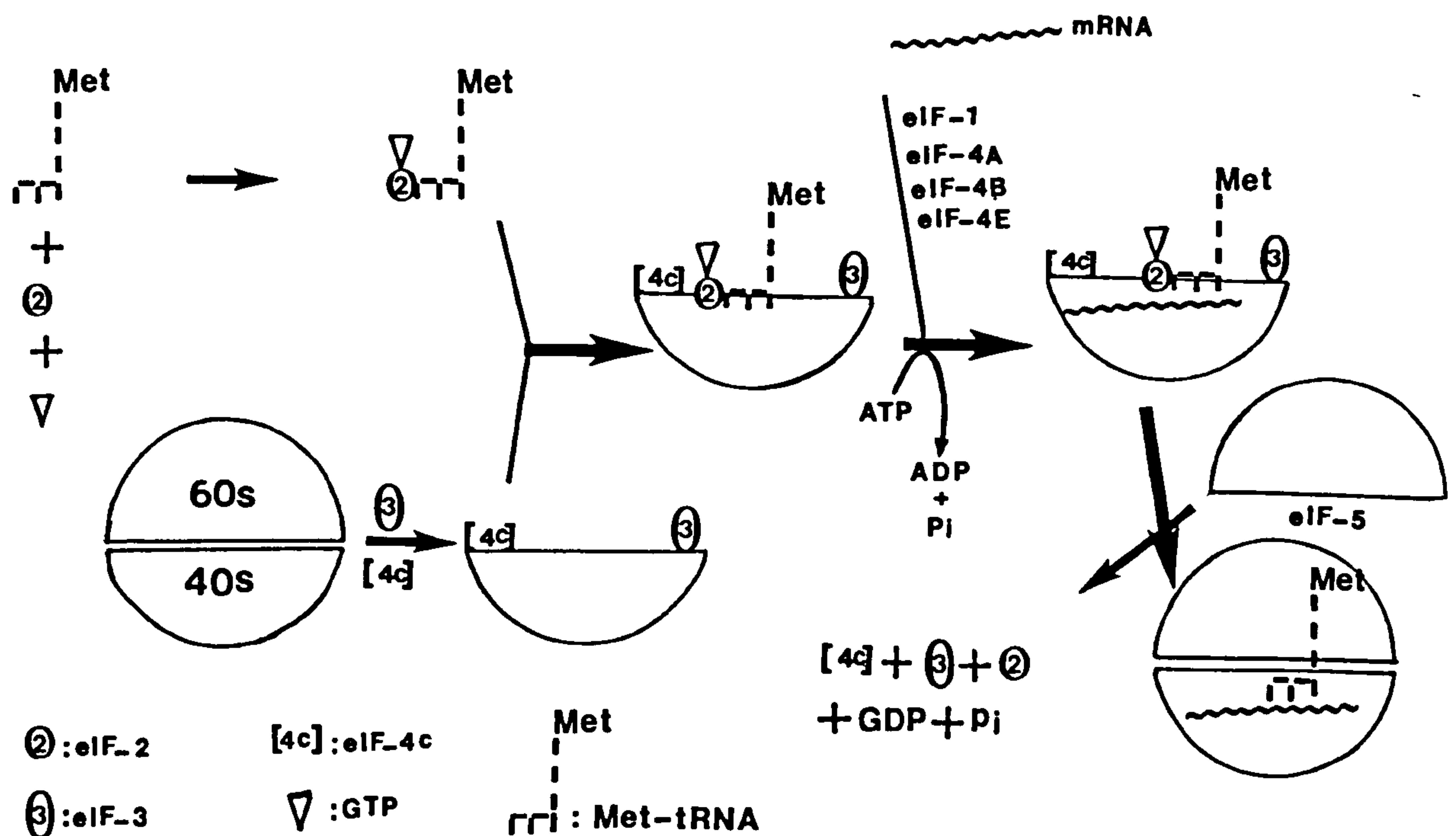
outcome. It is still possible that a selective protein that binds to the DNA at one or the other poly(A) site might instead trigger the choice of poly(A) site during transcription (Darnell, 1982).

#### 1.2.4 Translational control

The initiation of polypeptide chain synthesis in eukaryotes involves a complex series of events, including a number of nonribosomal initiation factors (eIF) (for review see Thomas et al, 1981)

The primary event in the initiation of protein synthesis is the formation of a ternary complex (80S. Met-tRNA.mRNA) and the first important step in the formation of this complex is the association of eukaryotic initiation factor (2) (eIF-2), which consists of three subunits ( $\alpha, \beta$ , &  $\gamma$ ), with met-tRNA and GTP molecules (Gupta et al, 1973). Factor eIF-2 as well as having a crucial role in this association is subjected to regulation by phosphorylation (Clemens et al, 1982; Pain & Clemens, 1983). A summary of the initiation of protein synthesis is shown in Figure (2).

Figure 2      Initiation of protein synthesis (adapted from Thomas et al, 1981)



Gene expression in eukaryotes often involves the selective translation of certain mRNA templates over the others. This type of regulation is used frequently in virus-infected cells and in cell differentiation. Most animal viruses are able to adopt the protein synthesising machinery and make it translate the viral mRNA and shut-off the translation of the host mRNA. This mRNA discrimination is thought to occur mainly at the initiation step which involves the recognition of mRNA and binding of the ribosome.

It has been observed that Mengovirus RNA competes thirty five times more effectively than globin mRNA, in the reticulocyte lysate system, for a critical component in translation (Rosen et al, 1982). The competition is relieved by the addition of eIF-2. This suggests that the virus RNA competes directly or indirectly with the globin mRNA for eIF-2. In another experiment Walden et al (1981), using reovirus infected SC-1 cells, have found that viral and host mRNAs must compete for a message-discriminatory component prior to their binding to the 40S ribosomal complex. However, they also found that the host mRNAs in general have greater affinities than reovirus mRNA.

Among the other possible control mechanisms for gene expression at the translational level in eukaryotes, is the sequestering of mRNA into inactive particles (mRNPs) unassociated with ribosomes (Heywood & Kennedy, 1976; Havaranis & Heywood, 1981). In addition, Bester et al (1975) have suggested that a small RNA named translational-control RNA (tcRNA) is involved in maintaining the mRNA in an inactive state. It has been reported recently that small RNAs are associated with stored mRNPs in a variety of cell systems (Vincent et al, 1980; O'Loughlin & Gross, 1981). Indeed a myosin heavy chain (MHC) mRNA has been purified and shown to contain a number of low molecular weight RNAs (Havaranis & Heywood, 1981). Recently, three low molecular weight RNAs molecules including tcRNA have been found in chick embryonic skeletal muscle. The



tcrNA (102 nucleotides) has been shown to inhibit the translation of mRNAs with which it is associated (McCarthy et al, 1983). Interactions of mRNA-tcrNA 102 alter the secondary structure of mRNA. Some kind of specificity is involved in this RNA-RNA interaction because tcrNA has shown no tendency to associate with ribosomal RNA or globin mRNA (McCarthy et al, 1983). This may suggest a certain degree of control at the translational level.

#### 1.2.5 Control at the Level of DNA Methylation

Modified purine and pyrimidine bases in DNA have been observed for more than three decades (reviewed by Razin & Riggs, 1980; Adams & Burdon, 1982). The most significant of these modifications is the methylation of cytosine at position number five (<sup>m5</sup>C) and to a lesser extent 6-methylaminopurine (<sup>m6</sup>A).

The methyl group in 5-methylcytosine is located in the major groove of DNA helix and does not change the base pairing characteristics of cytosine (Razin & Riggs, 1980). The methylated base <sup>m5</sup>C has been found in 2-7% of mammalian DNA (Vanyushin et al, 1970). Methylation occurs enzymatically after DNA synthesis by methyl transfer from S-adenosylmethionine (Burdon & Adams, 1969).

The studies on methylated sites have become intensified since the discovery of the inverse relationship between the sites of digestion of many bacterial endonucleases and the methylated sites of the genome (Razin & Riggs, 1980). Many bacterial restriction enzymes have shown a strong affinity for unmethylated restriction sites but they have low affinity and no activity on methylated sites (Smith, 1979; Roberts, 1978).

The location of the methylated cytosine is non-random. About 90% of <sup>m5</sup>C residues in eukaryotic DNA are found in the dinucleotide sequence CpG. Several restriction enzymes include CpG in their

recognition sequence (Roberts, 1978) and most of these enzymes do not digest the DNA if the CpG sequence is methylated (Van der Ploeg & Flavell, 1980).

The preliminary indication that gene activity was associated with undermethylation was reported by Christman et al in 1977. This finding has been supported later by the work of Jones and Taylor (1980).

However, Rogers and Wall (1981) working on immunoglobulin heavy chain genes in relation to methylation and class switching, have noted that the  $\mu$ ,  $\gamma_1$ , and  $\delta$  genes are all methylated when they are expressed.

Numerous reports correlating hypo- and hyper-methylation with the state of gene activation have been published recently. The restriction sites of digestion of some endonucleases are similar but can differentiate between the methylated and unmethylated CG sequence (Jones et al, 1981; Piekarczyk, 1982). In assessing the relationship between the state of methylation and gene activation, some groups have concluded that in mammalian sperm, RBCs, and early embryos, the DNA is highly methylated and undermethylation is associated with gene activity (Waalwijk & Flavell, 1978; Mandel & Chambon, 1979; Jones et al, 1981; Bird et al, 1981) while in contrast, unmethylation at the HpaII sites in a variety of tissues including sperm has been noted (Bower et al, 1983). However, co-existence of methylated and unmethylated sequences in the same genome has been observed in a wide range of eukaryotes. In addition, the presence of unmethylated sequences in the otherwise heavily methylated genomes of vertebrates suggests that methylation compartments are a general feature of eukaryotic genomes (Cooper et al, 1983).

Naveh-Manny & Cedar (1981) have suggested that transcribed sequences in the chicken are undermethylated compared to those in the genomic DNA. However, analysis of individual chicken genes has shown a rather complex picture with methylation patterns that vary for different genes. Moreover, Weintraub's group has shown a correlation between the



DNase-I sensitivity, the presence of HMG-14 and -17 and acetylated histones, a deficiency of  $m^5C$  at certain sites and the expression of globin genes during the development of the chick (Groudine & Weintraub, 1981; Weintraub et al, 1981). Weintraub et al (1981) have analysed the chromosome structures and expression of  $\alpha$ -gene cluster in developing chicken embryos and have shown that adjacent regions at both 5'- and 3'-sides of the active  $\alpha$ -genes are methylated and relatively insensitive to DNase-I. As a general conclusion a correlation between regions of the chromosomes that are undermethylated and regions that are expressed seems to be favoured.

#### 1.2.6 Control at the Level of Chromatin Structure

It is now widely accepted that active genes are packaged into an altered nucleosome structure. The chromatin structure of the transcribed genes is different from that of the bulk of DNA. The difference reflects an essential feature in explaining some aspects of gene expression, especially with respect to nuclease digestion. Other variations which may dictate special function in gene activity stems from the role of some non-histone proteins and variant histone components.

##### 1.2.6.1 Nuclease Sensitivity

The use of DNases has helped a great deal in understanding and differentiating the areas of actively transcribed gene from the inactive ones. Weintraub & Groudine (1976), studying transcribed and non-transcribed genes have demonstrated that the globin gene in chick erythrocyte nuclei is preferentially sensitive to digestion by pancreatic DNase-I. They have also shown that the gene is resistant to micrococcal nuclease which suggests that it is packaged into nucleosome-like particles that are different from most other nucleosomes. The DNase-I sensitivity has been found to be tissue specific (Weintraub & Groudine, 1976; Garel & Axel, 1976). DNase-I sensitivity of actively transcribed genes seems to be a general phenomenon (Garel & Axel, 1976; Flint &

Weintraub, 1977; Groudine et al, 1978; Storb et al, 1981). However, the micrococcal nuclease sensitive fraction of calf thymus nucleosomes have also been found to be enriched in transcriptionally active DNA (Davie & Saender, 1981). In a similar fractionation technique and using micrococcal nuclease, it has been possible to separate actively transcribed genes and genes which are transcribed at different rates. Results obtained by this method have been difficult to substantiate (Weisbrod, 1982).

Recently, Nicolas et al (1983) have demonstrated that higher order chromatin structures might be responsible for nuclease sensitivity of active genes. Gottschling et al (1983) have reported that the transcribed region of rRNA genes in Tetrahymena has a shorter nucleosome repeat compared with the non-transcribed central spacer of bulk chromatin. However, the transcribed region has been found to display an increased sensitivity to micrococcal nuclease in rapidly growing cells which suggests an altered chromatin structure during transcription. In an extension to the previous finding, Palen and Cech (1983) have concluded that transcriptional activation is accompanied by major changes in the structure of the ribosomal gene chromatin and that the extent and/or type of structural alteration differs in each functionally defined region of the rDNA.

DNase-I sensitivity as a property of active gene regions has been correlated with under-methylation of DNA (Razin & Riggs, 1980). This correlation has been noted in the chicken globin gene (Weintraub et al, 1981), ovalbumin (Kuo et al, 1979) and in other systems (for review see Adams & Burdon, 1982).

DNase-I hypersensitive regions have also been observed. These are located in specific positions of the genome. In Drosophila, they are located in the vicinity of the heat shock genes (Wu, 1980) adjacent to the 5'-ends of the coding regions. Similarly, DNase-I hypersensitive



sites have been found in other systems (Stadler et al, 1980; Weintraub et al, 1981), also situated adjacent to the coding sequences of the genes. Tissue specificity of DNase-I hypersensitivity has been found in rat preproinsulin-II gene system, predominant in insulinoma and absent in nonexpressing tissues such as kidney, spleen and brain (Wu & Gilbert, 1981). The structural basis and the functional significance of DNase-I hypersensitivity are not well understood (see Igo-Kemenes et al, 1982).

#### 1.2.6.2 Histone H<sub>1</sub>

Chromatin is usually undergoing extensive transient alterations in structure during the cell cycle. During (S) phase, the DNA must be made accessible in such a way to be presented to DNA-polymerases. Later during mitosis, the DNA is folded and compacted to form the mitotic chromosomes. Several structural and functional protein changes must accompany this sequential organisation. H<sub>1</sub> histone is believed to play a role in the higher order structure of chromatin (see section 1.1.1.2). The occurrence of precisely timed stage-specific changes in histone subtypes during development suggests that they are important in differentiation (Cohen et al, 1979). Direct evidence for the specific exchange of one histone subtype for another has been demonstrated in fertilised sea urchin eggs (Poccia et al, 1981). The switch in histone H<sub>1</sub> occurs almost immediately while the core histones are replaced at specific intervals.

Post-synthetic histone modification by phosphorylation, acetylation, methylation and poly ADP ribosylation is a well studied feature of chromatin structure and metabolism. Attempts have been made to link these modifications to transcriptional activation. Correlations between an increase in histone acetylation and increased RNA synthesis have been observed (Weisbrod, 1982). Special attention has been focu sed on linking the enrichment of histone-acetylation to transcriptionally active chromatin (Weisbrod & Weintraub, 1981).

Phosphorylation of H<sub>1</sub> histone has been implicated in gene activation. H<sub>1</sub> is generally present in 2-5 subtypes differing from one another in the N-terminal region of the molecules (for review see Cartwright et al, 1982). H<sub>1</sub> histone undergoes cell cycle related phosphorylation at several sites, principally during (S) phase and mitosis (Hohmann et al, 1976; Ajiro et al, 1981a, 1981b). These observations have suggested that H<sub>1</sub> phosphorylation plays a part in some of the conformational alterations of chromatin during the cell cycle. It has also been shown that within a single cell type there are differences between H<sub>1</sub> subtypes with respect to the extent of phosphorylation during the cell cycle (Hohmann et al, 1976; Ajiro et al, 1981a, 1981b). However, H<sub>1</sub> subtypes differ from each other functionally (Lennox et al, 1982). It has also been demonstrated that there are qualitative differences between the H<sub>1</sub> complement of undifferentiated embryonal carcinoma cells and differentiated endodermal cells, raising the possibility that such changes may play a role in the process of cell differentiation (Lennox et al, 1982).

#### 1.2.6.3 High Mobility Group (HMG) Proteins

High mobility group proteins are low molecular weight non-histone chromosomal components. They have been found to possess common features in a number of chromatin preparations of various origins (Goodwin et al, 1973; Alfageme et al, 1976; Spiker et al, 1978). The most important proteins of this group are HMG-1, -2, -14 and -17. Their importance stems from the possible structural and functional roles of these proteins. Analysis of nucleosome preparations has shown that HMG-17 and -14 are found in association with mononucleosomes (Mathew et al, 1979), in particular binding DNA sequences between 145-160 bp as well as within the 145 bp core particle (Goodwin et al, 1979). However, Sandeen et al (1980) have shown that major sites of HMG-14 and -17 binding are located near the termini of the core particle DNA. It has



also been reported that HMG-14 and -17 bind to a well defined nucleosomal structure rather than to DNA (Weisbrod et al, 1980).

Chromatin fractionation and nuclear digestion have been used to differentiate between proteins associated with active and inactive genes. Rapidly released and/or soluble nucleosomes are generally reported to be enriched in transcribed DNA sequences and in HMG-14 and HMG-17 (Levy-Wilson & Dixon, 1978; Mathew et al, 1979), and small amounts of HMG-1 and HMG-2 are also found (Jackson & Rill, 1981; Goodwin et al, 1979; Nicolas et al, 1983). However, several reports have correlated the association of HMG proteins with the maintenance of actively transcribing regions in chromatin (Levy-Wilson et al, 1977; Vidali et al, 1977).

The functional connection between HMG-14 and -17 and the active genes has been well demonstrated by the experiment of Weisbrod and Weintraub (1979). In this experiment it has been shown that DNase-I sensitivity of globin genes in erythrocyte chromatin was lost on the removal of HMG proteins with 0.35M NaCl. Similar findings have been reported by Nicolas et al (1983) working on chick embryonic red blood cells. The sensitivity to DNase-I has been restored by reconstitution with HMG-17 and HMG-14 (Weisbrod & Weintraub, 1979; Gazit et al, 1980; Nicolas et al, 1983). Weisbrod et al (1980) have studied the interaction of HMG-14 and -17 with actively transcribed genes by monitoring the sensitivity of specific genes to DNase-I after reconstitution of HMG-depleted chromatin with HMG-14 and HMG-17. They have observed that the most actively transcribed genes become sensitised to DNase-I by HMG-14 and HMG-17. Both HMGs have activity which can be reconstituted onto isolated nucleosome core particles. Furthermore, HMG-14 and HMG-17 have been found to bind equally well to genes transcribing large amounts of mRNA and to genes that are transcribed rarely.

Further evidence supporting the correlation of the presence of HMG-14 and HMG-17 with gene activity, stems from the altered rate of phosphorylation at different stages of cell cycle. Bhorjee (1981), has demonstrated a seven fold increase in  $^{32}\text{P}$  incorporation into HMG-14 in (G2) phase of the cell cycle compared with (G1), and a two fold increase in incorporation into HMG-17 in early (S) phase. This report has also concluded that the differential phosphorylation of HMG-14 and -17 at specific stages of the cell cycle suggests that they may well play a role in tissue-specific maintenance of the altered chromatin structure characteristic of potentially active or actively transcribed chromatin domains.

In another significant finding, the stage-specific phosphorylation of HMG-14 has been found to be parallel to that of histone  $\text{H}_1$ , and this adds weight to the theory that HMG-14 and HMG-17 substitute for  $\text{H}_1$  in active regions of chromatin (Weisbrod, 1982).

### 1.3 Nonhistone Chromosomal Proteins

The nonhistone chromosomal (NHC) proteins are a class of highly heterogeneous proteins in eukaryotic nuclei. Some of them are assumed to play a crucial role in the mechanism of gene activity. They can be divided into three groups, based on their affinity for DNA, the low, intermediate and high-affinity proteins. The high mobility group (HMG) proteins fall within the DNA-low affinity group. The comparatively straight forward methodology of their isolation and purification, coupled with their abundance has led to fairly detailed characterisation of the principal members of this group and tentative structural and functional roles have been suggested. In contrast, the high-affinity proteins have proved difficult to isolate and characterise and therefore little is known regarding their roles. However, attempts have been made to determine the role of some isolated proteins which have intermediate-affinity for DNA. Amongst these proteins are enzymes and a



number of cofactors. In addition, a number of chromosomal proteins with no known enzymatic or enzyme-associated activity have been identified and resolved from the bulk of chromosomal proteins by electrophoresis. The lack of known enzymatic functions and the large amounts of each present in a given cell, suggest that these proteins are involved in structural organisation. Another group of proteins are found to be species-, tissue- and cell-specific. Quantitative variation of a number of these has been associated with genetic activity in some cells. These groups of proteins are analysed in greater detail in the following sections.

#### 1.3.1 Enzymes and Co-factors

A number of enzymes essential for the metabolism of nucleic acids and protein modification have been localised in the nonhistone fraction of chromosomal proteins. These include enzymes involved in DNA, RNA, histone and nonhistone protein metabolism and modifications. Examples of the different components with their assigned functions are summarised in Table (1).

Initial attempts to examine the role of NHCPs in the regulation of transcription were made by assessing the effects of these proteins on DNA-dependent RNA synthesis. Results from several laboratories indicate that NHCPs added directly to histone-DNA complexes of various chromatin preparations, increase the availability of DNA as a template for RNA transcription (Kaniyam & Wang, 1971; Spelsberg & Hnilica, 1971a & b). A protein co-factor which stimulates RNA polymerase I has been found to be crucial for transcription of native calf thymus DNA (Goldberg et al, 1977). Le Graverend and Glazer (1980) have characterised a nonhistone chromosomal protein from rat liver nuclei which stimulates RNA synthesis in vitro.

In addition, isolation of RNA polymerase II stimulatory factors from calf thymus chromatin (Benson et al, 1978), lamb thymus (Revie & Dahmus, 1979), Ehrlich ascites tumour (Natori et al, 1973), rat liver

**Table 1**  
**Enzyme Components of Nonhistone Chromosomal Proteins**

Enzyme/Function	Source	Reference
1) <u>Nucleic Acids &amp; Nucleotides as substrate:</u>		
a. RNA polymerase	Hen-oviduct	Cox (1973)
	Rat liver	Tata & Baker (1974)
b. poly A polymerase	Wheat seedlings	Sasaki & Tadashi (1973)
c. DNA polymerase	Mouse ascitic cells	Schlaeger et al (1978)
	Rat ascitic hepatoma	Tsuruo & Ukita (1974)
	Rat liver	O'Connor (1969); Chan et al (1977)
d. DNA endonuclease	HeLa cells	Urbaczynski & Studzinski (1974)
e. DNA methylase	L929 cells	Qureshi et al (1982)
f. DNA ligase	Rabbit bone marrow	Gaziev & Kuzin (1973)
g. Alkaline deoxyribonuclease	Rat liver	O'Connor (1969)
h. Terminal DNA-nucleotidyltransferase	Calf thymus	Wang (1968)
i. Nicotinamide mononucleotide adenyl transferase	Chick erythrocytes	Cantarow & Stoller (1977)
2) <u>Chromosomal Proteins as substrate:</u>		
a. poly ADP-ribose polymerase	HeLa cells	Mullins et al (1977); Butt et al (1978); Nolan et al (1980)
	Rat liver	Yukioka et al (1978)
	Calf thymus	Ito et al (1979)
b. poly ADP-ribose glycohydrolase	Rat liver	Miyakawa et al (1972)
c. Deacetylase	Calf thymus	Vidali et al (1972); Krieger et al (1974)
	Lymphocytes	Böhm et al (1980)
d. Histone specific acetyltransferase	Calf thymus	Sures & Gallwitz (1980)
	Calf liver	Libby (1978)
e. Histone & spermidine acetyltransferase	Bovine thymus	Shoemaker & Chalkley (1980)
f. Histone kinase	Rat liver	Ramponi et al (1978); Watson & Moudrianakis (1982)
g. Histone protease		Hagiwara et al (1980)
	Normal/tumour rat tissues	Hagiwara et al (1980)
h. NHC protein protease	Normal/tumour rat tissues	Takeda et al (1971)
i. NHC protein kinase	Rat liver	Böhm et al (1977)
j. Protein kinase	Mouse ascitic cells & bovine lymphocytes	Comb et al (1966)
k. Protein methylase	Ehrlich ascites carcinoma	



(Seifart et al, 1973) and mouse myeloma cells (Lentfer & Lezius, 1972) has also been reported. Recently, an NHCP-fraction has been chromatographically isolated and found to contain a large amount of nascent RNA chains (Ferraro et al, 1982).

Three factors associated with RNA polymerase III have been purified from human KB cells (Segall et al, 1980), and Xenopus (Pelham & Brown, 1980). Factors from KB cells have also been found to stimulate accurate initiation of transcription at the major late promoter of Adenovirus-2 (Matsui et al, 1980).

### 1.3.2 Structural Proteins

HMG proteins -14 and -17 have been found to bind to the core nucleosome and have a major effect on nucleosome structure (Mathew et al, 1979). Reconstitution experiments have indicated that both HMG-14 and -17 bind readily to the 145-base pair core particle at a stoichiometry of 1 and 2 molecules per core, respectively, in a manner that does not exclude binding of  $H_1$  (Albright et al, 1980). Another protein of this group is HMG-20 (Ubiquitin) (Schlesinger et al, 1975) which has been suggested to reside on linker DNA, between the core particles, in the transcriptionally active chromatin (Watson et al, 1978). Ubiquitin, however, is found as a constituent of the conjugate protein A24 which consists of  $H_2A$  histone and HMG-20 (Goldknopf & Busch, 1977). HMG-20 is covalently linked via a glycyl-glycine peptide at its C-terminal end to the amino group of lysine -119 in histone  $H_2A$ . The absence of ubiquitin in mitotic chromatin, has led to the suggestion that it plays a structural role within the chromatin components preventing condensation to higher-order structures in metaphase (Matsui et al, 1979). Recently, A24 has been found to be associated with mononucleosomes containing transcriptionally inactive sequences (Levinger et al, 1981).

Detke and Keller (1982), in their comparison between the interphase nucleoskeleton and metaphase chromosome scaffolds in HeLa

cells, have found that the major proteins of both preparations are similar. They have also distinguished the nucleoskeletal proteins from the major nuclear laminar proteins. Although the nucleoskeletal components comprise only a small subset of the total nuclear proteins, it has been suggested that they are responsible, at least in part, for the gross morphology of the nucleus (Riley et al, 1975).

Another group of proteins which may play a part in the structural interconversions of chromatin as well as in the regulation of cell proliferation are the contractile proteins (LeStourgeon et al, 1975). These proteins have been found in the NHCP-fraction, and suggested to be involved in intranuclear contractile functions.

The structure of metaphase chromosomes is stabilised by another class of NHC-proteins, the tightly-bound scaffold proteins (Adolph et al, 1977 a & b). Accumulated evidence suggests that both metaphase chromosomes and nuclei contain a residual skeleton or scaffold which maintains the DNA in a compact, looped arrangement (Laemmli et al, 1978). Two types of scaffold protein fractions have been characterised, based on their method of preparation (Lebkowski & Laemmli, 1982). The type-I structure has been shown to be stabilised by the  $\text{Ca}^{++}$  ion. This stabilisation can be altered by the addition of 2-mercaptoethanol which disrupts the metalloprotein interaction to give a type-II structure. The metalloprotein interaction involved is suggested to be important for DNA folding. DNA binding studies have revealed that twelve proteins of type-I and four of type-II scaffolds bind DNA in vitro (Lebkowski & Laemmli, 1982). However, it has been shown that following treatment with cupric ion, the scaffold residue shows a simple protein composition comprising two high molecular weight (135K & 170K) proteins. They are designated as scaffold 1 and 2 (Sc1 & Sc2) respectively (Lewis & Laemmli, 1982). More recently, Earnshaw and Laemmli (1983) have visualised a nonhistone protein residual scaffold by



electron microscopy. This protein scaffold has been obtained from nuclease digested chromosomes under conditions of low salt protein extraction. It is suggested that the extremely simple composition of the residual scaffold proteins, is fibrous in nature and determines the dimensions of the chromosomes. Evidence has been presented to demonstrate that the scaffolding network plays a dynamic role in chromosome condensation at mitosis (Earnshaw & Laemmli, 1983).

### 1.3.3 Tissue and Cell Specific Proteins

Several attempts have been made to correlate the presence of nonhistone chromosomal proteins with certain specific tissues or cell types. Comparison between the chromosomal proteins of a number of tissues and cells has been performed by immunological criteria or electrophoretic profiles. A fraction of nonhistone proteins has been found necessary for the in vitro transcription of tissue-specific RNA species (Spelsberg et al, 1971). A dehistonised chromatin fraction from Novikoff hepatoma has been shown, by immunological criteria, to be tissue specific (Chytil & Spelsberg, 1971), and to require and recognise only homologous DNA (Wakabayashi & Hnilica, 1973) for its immunoactivity. In comparison between tumour and normal tissues, Fujitani et al (1978), have localised a protein antigen (45K - 60K) to the chromatins of embryonic liver and several transplantable tumours but not of normal tissues. Further immunological studies on the Novikoff hepatoma antigens have revealed the presence of two proteins (P39 & P 49) specific for Novikoff hepatoma. These antigens have been localised in chromatin as well as in the cytoplasm (Schmidt et al, 1981). A similar approach has been used on the nonhistone chromosomal protein fraction from HeLa cells. The HeLa antigens have been identified in the DNA-tightly-bound proteins in the scaffold area (Campbell et al, 1979). This group of antigens has been found by immunological criteria to be cell specific (Briggs et al, 1979) and species specific (Dunn et al, 1980). However, a different

110K nonhistone phosphoprotein purified from the 0.35M NaCl nuclear extract has also been found common to four cell lines including Novikoff hepatoma and HeLa cells but failed to be traced in normal tissues (Durban et al, 1981).

Electrophoresis has been used to demonstrate and characterise specific proteins in different chromatin preparations. Yaneva et al (1980) have shown, by electrophoretic comparison, the presence of at least one unique band in each tissue examined. Quantitative and qualitative differences in the electrophoretic profiles have also been noted. Two dimensional gel electrophoresis has been used by Wu et al (1981) to demonstrate differences in the pattern of proteins of several human tumours and normal cells. Two proteins (140K & 54K) have been found in four types of tumour cell but not in the four normal cell types examined, whereas another two proteins, of 56K but different isoelectric points, have been shown to occur only in the normal cells and not in the tumour cells. Qualitative and/or quantitative differences in the two dimensional gel patterns of NHCPs have also been observed in closely related mouse cell lines (Plasmacytomas) which were known to differ in their ability to express known genes (Lincoln & Stott, 1983). These differences were correlated with gene expression.

#### 1.4 Characterisation of Nuclear and Nucleolar Components by Immunological Approaches

##### 1.4.1 Antibodies to Nucleic Acids

Antibodies to purified nucleic acids are obtained with great difficulty. Several reports have described successful immunisation with RNA preparations. Antisera obtained from rabbits immunised with yeast RNA have shown reactivity towards yeast, human, rabbit and rat RNA (Lamon & Bennett, 1970). Hernandez et al (1968) have shown that after immunisation of four rabbits with tRNA, believed to be free from protein carriers, and twenty nine rabbits with oligonucleotides prepared by



partial enzymatic digestion of RNA, almost all antisera obtained from both types react in the complement fixation assay with RNA of high molecular weight as well as with oligonucleotides prepared from tRNA. However, one antiserum out of 33 reacts specifically with tRNA itself. On the other hand, antisera obtained after immunisation with free tRNA have failed to react with tRNA (Bonavida et al, 1970).

Despite the difficulties experienced by immunisation of experimental animals, anti-RNA antibodies are commonly detected in autoimmune diseases in man and animals. Anti snRNA antibodies have been detected in sera from patients with SLE (Lerner et al, 1980, 1981). Successful hybridomas with reactivity against rRNA have been produced from autoimmune mice (Eilat et al, 1980). One of these monoclonal antibodies derived from NZB/NZW ( $F_1$ ) mouse lymphocytes fused with the myeloma cell line MPC-11, has shown specific binding to rRNA and failed to bind significantly to DNA, tRNA and synthetic single- and double-stranded polynucleotides. Similarly, monoclonal antibodies derived from MRL mice have shown specific reactivity towards rRNA (Lerner et al, 1981). Eilat et al (1982) produced a sequence specific anti-RNA monoclonal antibody which recognise a G/C-rich ribonucleotide.

Antibodies to DNA have been proved difficult to raise in laboratory animals. Antisera from animals immunised with purified DNA from calf thymus, salmon sperm, E.coli and rat liver have also failed to show immunoreactivity towards DNA (Levine & Stollar, 1968; Barnett & Vaughan, 1966; Yachin, 1962). This led Stollar (1973) to conclude that native DNA is not immunogenic.

However, spontaneous antibodies reacting with native DNA have been reported in almost all patients with Systemic Lupus Erythromatosus (Tan et al, 1966). Evidence for the presence of antisera which react with ss-DNA in patients with SLE and other autoimmune diseases has been presented (Koffler et al, 1973, 1974). Specific antisera which react



only with double-stranded DNA and others that bind both ds- and ss-DNA have also been demonstrated in SLE patients (Gilliam et al, 1980). Antisera which react with ds-poly d(AT) have been shown to bind well to ss-DNA (Gilliam et al, 1980). Peripheral blood lymphocytes from patients with no autoimmune disease show no production of anti-DNA antibodies but after EBV transformation (Epstein-Barr Virus), anti-DNA antibodies producing cells have been detected (Hoch et al, 1983). In addition, it has also been shown that normal mice can produce anti-DNA antibodies if their B-lymphocytes undergo mitogen-stimulated polyclonal activation (Fish & Ziff, 1982; Dziarski, 1982). Moreover, production of autoantibodies by human-human hybridomas has been reported from many laboratories. Shoenfeld et al (1982) using the ELISA technique have demonstrated the feasibility of producing anti-ss-DNA monoclonal antibodies using peripheral blood cells from a number of patients with autoimmune diseases such as SLE and Atopic Allergy. Recently, Shoenfeld et al (1983) have managed to fuse a human lymphoblastoid cell line derived from patient with multiple myeloma with lymphocytes from an SLE patient. Among the thirty monoclonal antibodies produced against denatured DNA, eighteen of them react with additional polynucleotide such as native DNA, left handed double helical DNA (Z-DNA), poly(I) and poly(dT). Ten of these monoclonal antibodies showed reactivity towards nucleic acids and cardiolipin phospholipid. Accordingly, this report suggests that DNA itself need not be the immunogenic stimulus for autoantibody formation in this type of disease.

Laboratory animals such as NZB/NZW mice have also been found to make antibodies to DNA (Stollar, 1973). Lee et al (1981) have produced six hybridomas which secrete extremely specific monoclonal antibodies to deoxyribonucleic acids from NZB/NZW mice. It has been shown that various RNAs failed to react with these monoclonal antibodies. In addition, the ss-DNAs tested showed higher reactivity than ds-DNA. Some

of these monoclonal antibodies exhibit sequence preferences which make them a powerful tool for distinguishing between similar nucleic acids. Other groups have succeeded in producing a number of hybridomas that secrete monoclonal anti-DNA autoantibodies from NZB/NZW (Hahn et al, 1980; Jacobs & Tron, 1982) or MRL mice (Lerner et al, 1981). Hahn et al (1980) in their studies using murine anti-DNA monoclonals have shown that these antibodies exhibit specificity towards ds-DNA and ss-DNA but not ss-RNA or poly(I-C). Jacobs and Tron (1982) and Andrzejewski et al (1981) produced 10 anti-DNA monoclonal antibodies with identical specificity to a conformational determinant of the B-helical form of ds-DNA. Other anti-nucleic acid monoclonal antibodies have recently been reported (Kioke et al, 1982). These monoclonals were produced from hybrids of (MRL 1) X NZB-NZW ( $F_1$ ) mouse lymphocyte fused with 653 myeloma cell line. Twelve clones have shown specific reactivity with ss-DNA only while another two react with both ss- and ds-DNAs. Studies with mouse anti-DNA monoclonal autoantibodies have revealed that a single antibody can bind to multiple nucleic acid antigens of different base composition (Andrzejewski, 1981; Pisetsky, 1982; Kioke et al, 1982). Structural differences in polynucleotide backbones can explain individual variations in binding specificities among the monoclonal antibodies. In addition, phosphodiester-containing epitopes also account for the ability of some monoclonal anti-DNA autoantibodies to bind to certain phospholipids such as cardiolipin (Lafer et al, 1981c). Immunisation of normal rabbits (Guarnieri & Eisner, 1974) or mice (Rauch et al, unpublished data) with cardiolipin has been found to stimulate the production of both anti-cardiolipin and anti-DNA antibodies. Moreover, MCAs derived from these cardiolipin-immunised mice, bind to cardiolipin, DNA, poly(I) and poly(dT). The polyspecificities of some MCAs signify that it is not possible to imply the origin of spontaneously produced autoantibodies from serum analyses only.



In contrast to most other nucleic acids, left-handed-DNA (Z-DNA) is a strong immunogen which elicits Ab production in both mouse and rabbit. The anti-left handed-DNA Abs react with only Z-DNA and show no reactivity with native B-DNA (Lafer et al, 1981a). However, nuclei of many rat cells exhibit Z-DNA immunoactivity with anti-Z-DNA which suggests that Z-DNA may exist naturally in mammalian chromatin (Morgenegg et al, 1983). In Drosophila, fluorescent staining with these Abs has shown specific binding to polytene chromosomes especially in the interband regions (Lafer et al, 1981b). Monoclonal antibodies to Z-DNA have also been produced recently and shown to exhibit high specificity (Pohl et al, 1982).

#### 1.4.2 Antibodies to Histones

Most of the amino acids sequences of histone proteins are similar in different species reflecting the conserved primary structures of these proteins. As a result of this fact, these proteins are in general weak antigens.

Antisera raised against total rat liver chromatin have shown no reactivity towards pure histone fractions, while antisera to purified histones failed to interfere with the reaction between antibodies to chromatin and chromatin itself as judged by C'F assay (Goldblatt & Bustin, 1975). Antisera raised against calf thymus H<sub>2</sub>A, H<sub>2</sub>B and H<sub>4</sub> histones have failed to distinguish between these histones and those obtained from chicken erythrocytes, frog liver and human spleen (Stollar & Ward, 1970). Pothier et al (1975) managed to raise antisera against the major histones of calf thymus. These antisera have been used to localise the distribution, by immunofluorescence of various proteins during the cell cycle, and also to follow their reactivity on chromosomes of different cells. Cross reactivity is common among all but H<sub>1</sub> histone. Antibodies elicited against H<sub>1</sub> and H<sub>2</sub>B histones of Drosophila have also been used coupled to fluorescent labels in order to



localise their positions on polytene chromosome (Alfageme et al, 1976).

Both  $H_1$  and  $H_2B$  have been found in most chromosomal regions.

In contrast to antibodies to the four other histones, those elicited against unfractionated  $H_1$  derived from calf thymus (Bustin & Stollar, 1972) or rat tissues (Hekman & Sluyser, 1973) have shown both species and organ specificity in this histone class. Similarly, specific antisera against  $H_1$ -subfractions have been used to detect species- and tissue-specificity (Sluyser & Bustin, 1974) as well as changes in conformational determinants and changes due to chemical modifications (Bustin & Stollar, 1973).

High levels of antibodies to histones have been found in many patients with autoimmune diseases. This makes the specific antisera a potentially useful diagnostic tool in such diseases. Anti-histone antibodies from sera of patients with various rheumatic diseases have been shown to react predominantly with histone complex  $H_3$ - $H_4$  (Rubin et al, 1982).

#### 1.4.3 Antibodies to Nonhistone Chromosomal Proteins

NHC proteins are the most heterogeneous group of nuclear proteins. The DNA-low affinity proteins are relatively easy to fractionate. A wide variety of antisera specific to components of this group has been reported. These have in general been used to locate the corresponding antigen in different subfractions or cell types. Bustin and Neihart (1979) have raised a rabbit antiserum to calf thymus HMG-1 and shown that it reacts with both the chromatin itself and also cytoplasmic components of Chinese hamster V-79, rat liver TR-12 and bovine trachea EBTr-NBL-4 cells. Another antiserum raised against chick oviduct HMG-1 has been used in a limited study of the effect of oestrogen stimulation on HMG-1 distribution in cells (Teng & Teng, 1981).

Antibodies to HMG-1 and HMG-2 proteins have also been detected by ELISA in antisera from patients with SLE and other autoimmune diseases (Bustin et al, 1982). However, the immune response to HMG-14 and HMG-17 proteins appears to be stronger.

The distribution of nonhistone proteins of a variety of molecular weights in polytene chromosomes of Drosophila salivary glands was visualised some years ago by the use of antisera raised to chromosomal antigens eluted from SDS-PAGE (Stumph et al, 1974). More detailed information on the location of a particular NHCP in the same polytene chromosome was later obtained using antiserum raised against purified D1 nonhistone protein (Alfageme et al, 1976). Similarly, antisera raised against other purified individual NHCP from Drosophila have been used to assess the selectivity of distribution patterns, and two of these have shown that the corresponding protein distribution is limited on the polytene chromosome (Silver & Elgin, 1978). On the other hand, an antiserum raised against a 21K protein has shown prominent immunofluorescent staining at dense chromomeres and the chromocentre in a pattern mimicking DNA distribution (Silver & Elgin, 1978).

The high affinity NHC protein group has proved more difficult to fractionate. This, together with the unknown function of most of these proteins, makes the isolation and identification of individual proteins more difficult. However, antiserum to a chromosomal protein fraction specific for HeLa cells has been elicited in rabbit. Although this antiserum has not been shown to react with a single protein, it has been useful to localise the immunoactivity to the transcriptionally inactive region of log phase chromatin (Campbell et al, 1979). Further studies have suggested that some of the antigenic proteins in the scaffold area are both tight binding and cell-specific. It has been shown that the specific antigens, of this chromatin fraction, are a complex of human or HeLa DNA only and the tightly bound NHC proteins. In addition,



immunochemical tests have revealed that the antigens are continuously present throughout the cell cycle (Briggs et al, 1979).

Another DNA:NHCP complex immunogen has been detected in Novikoff hepatoma (Wakabayashi & Hnilica, 1973). This immunogen has been shown to have species- and tissue-specificity (Fujitani et al, 1978). However, more recently antisera against three partially purified Novikoff hepatoma antigens from the same group have been used to localise their distribution in various subcellular fractions by immunoblotting techniques (Schmidt et al, 1981). These proteins, of 39K, 49K and 56K molecular weights have been found in cytoplasmic fractions as well as in isolated chromatin.

In an early approach, Chytil and Spelsberg (1971) showed that the antigenicity of NHCPs of chick oviduct chromatin was associated with DNA and was species-specific. In addition, the differential distribution of this antigen complex in different tissues indicated a tissue-specificity.

Species-specificity of another NHCP:DNA complex from chick reticulocytes have also been investigated by immunological criteria (Krajewska et al, 1979). Further immunological evidence for tissue-, species- and cell-specificity of this NHCP group have emerged from more recent studies. Erythroid cell-specific antisera detecting the tightly-bound DNA:NHCP complex from dehistonised chicken reticulocyte chromatin have shown no cross reactivity with chromatin of thrombocytes (Krajewska & Klyszejko-Stefanowics, 1982) although thrombocytes are closely related to erythroid cells. In addition, antisera to the tightly bound NHCP:DNA complex of thrombocytes have shown no immunological similarity with those raised against erythrocyte DNA-tightly bound chromatin. More recent studies have confirmed that antisera raised in mice to chromatin from different tissues of the



chicken reacted preferentially with the chromatin type that was used for immunisation (Vanderbilt & Anderson, 1983).

Antisera against a single NHCP of predefined function have also been used to determine the presence of such protein in different subcellular preparations. Watson and Moudrianakis (1982) have elicited an antiserum to an NHC protease. Antiserum against a nonhistone chromosomal phosphoprotein (B2) that is specifically associated with the nucleosome structure has also been reported (Zhao & Liew, 1982).

Monoclonal antibodies to NHCPs will be discussed in section (1.5.3).

#### 1.4.4 Antibodies to Nucleolar Antigens

The nucleolus is the site of synthesis of 28S, 18S and 5.8S ribosomal RNAs (Wellauer & Dawid, 1973). In addition to ribosomal RNA precursors, the nucleolus contains a small species of RNA, U<sub>3</sub> (for review see Ro-Choi & Busch, 1974). This species of RNA is of interest because of its unique and specific localisation in the nucleoli (Prestayko et al, 1971) and its hydrogen bonding to nucleolar 35S and 28S RNA (Prestayko et al, 1970). Other small RNAs can also be detected in nucleoli (reviewed by Busch et al, 1982). The assembly of ribosomes begins in the nucleolus. Therefore, it is likely that many of the molecular associations found in cytoplasmic ribosomes may also be present in nucleolar precursors.

Nucleolar immunofluorescent staining has been found in some SLE patients whose antisera contain antibodies against cytoplasmic antigen with rRNA characteristics (Miyachi & Tan, 1979). On the other hand, Homberg et al (1974) have described another type of distinctive immunofluorescent pattern found in some antisera of patients with SLE and other collagenous diseases. These sera which are of anti-ribosomal character have failed to react with nucleolar ribosomes (Bianchi et al, 1974).

In general, anti-nucleolar antibodies are more common in Scleroderma than in other SLE and Rheumatic diseases. Although earlier reports showed an 11-21% incidence of anti-nucleolar antibodies in Scleroderma patients (Fennell et al, 1962; Beck et al, 1963; Burnham et al, 1966), a higher percentage has been described in a later report (Ritchie, 1969). Anti-nucleolar antibodies have also been detected in Sjögren's Syndrome (Tan & Lerner, 1972) as well as in response to malignant melanoma (McBride et al, 1972).

Nucleolar specific antibodies have been raised in rabbits immunised with whole nucleoli from Novikoff hepatoma ascites cells or from normal rat liver. Of the fourteen antigens detected, ten reacted with both kinds of antisera (Davis et al, 1978). In addition, comparison of the nucleolar antigens of human liver and HeLa cells has shown a 54K immunogen in HeLa but not in normal liver cells (Chan et al, 1980). However, in a more detailed comparison, antisera raised against a nuclear 0.01M tris-HCl (pH 8) extract or nucleolar preparation of HeLa S3 cells, have shown a wide range of nucleolar immunofluorescence in 61 out of 63 human adenocarcinomas, squamous cell carcinomas, sarcomas, haematological neoplasms and other malignant tumours (Busch et al, 1979). With these antibodies, nucleolar immunofluorescence has not been visualised in 23 normal tissue specimens. In similar studies, Davis et al (1979) have also described the presence of a nucleolar antigen which was found in several human tumours but not in non-tumour tissues. These reports suggest that such specific antisera may be a useful tool for differentiating malignant from normal tissues.

## 1.5 Monoclonal Antibodies

### 1.5.1 History and Background

In 1975, Köhler and Milstein showed that somatic cell hybridisation could be induced in culture between antibody-producing B-lymphocytes which are mortal in tissue culture and myeloma cells which



continue to grow in appropriate conditions. The hybrid cell which results from such fusion has the ability to grow continuously and produce the B-lymphocyte specified antibody in selective culture media.

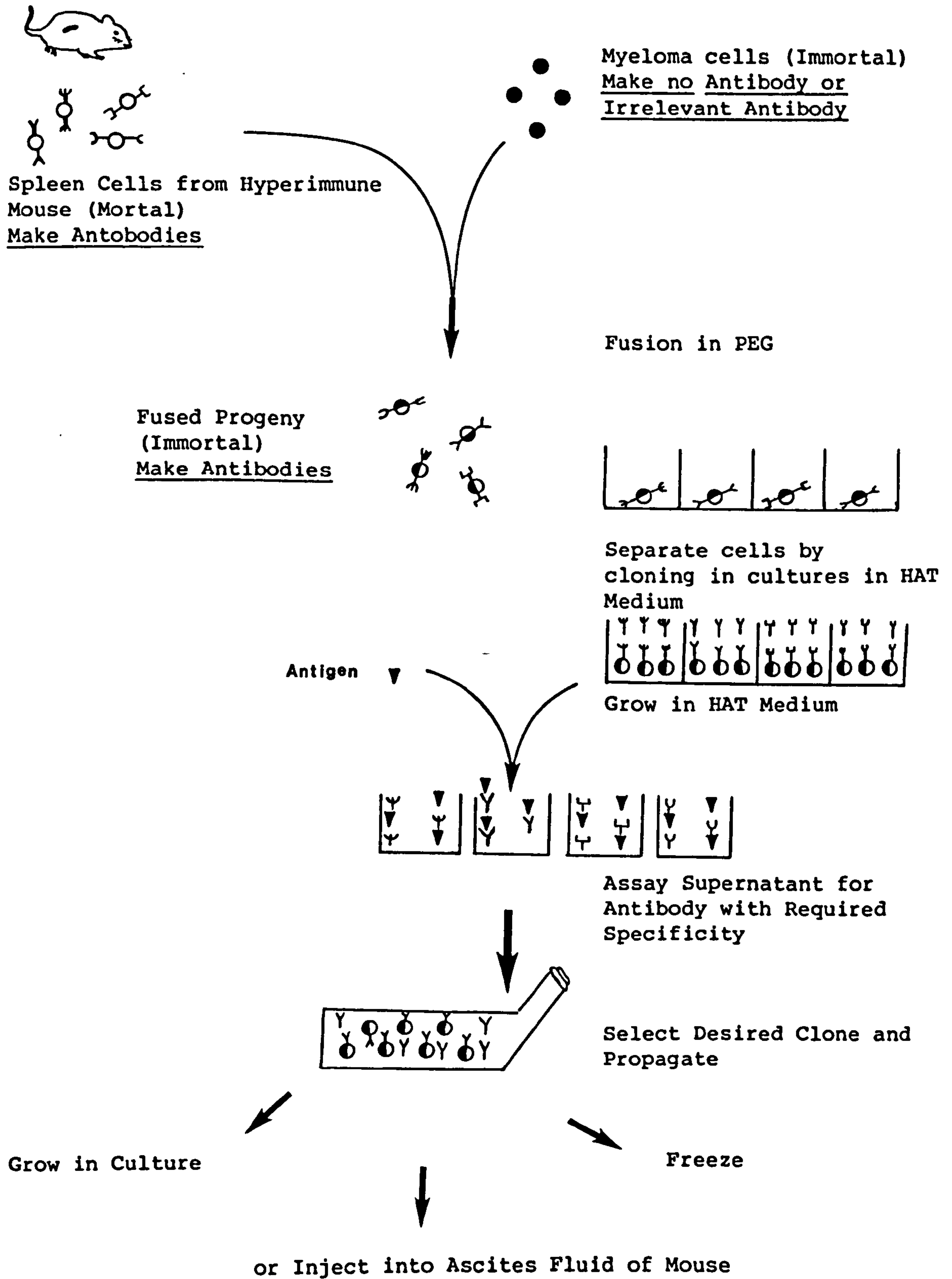
The two pathways of nucleotide biosynthesis in eukaryotic cells are the "De Novo" synthesis and the "Salvage" pathways. The De Novo synthesis of purines and pyrimidines can be blocked by the folic acid antagonist; Aminopterin. However, the cell can still synthesise DNA via the so called Salvage pathways, if it possesses the required enzymes, Thymidine Kinase (TK) for the pyrimidines, and Hypoxanthine Guanosine Phosphoribosyl Transferase (HGPRT) for the purines. When the De Novo synthesis of DNA is blocked, the cells which contain these enzymes can survive if exogenous hypoxanthine and thymidine are provided in the culture media (Littlefield, 1964). After the fusion event, three kinds of cells are mixed in the selective media, HAT media. The spleen cells which have the ability to synthesise the antibodies die in culture after a few days, while the myeloma cells which are usually either producer of irrelevant antibodies or non-producers, lack the enzyme HGPRT and thus cannot survive in aminopterin-supplemented media. Only the hybrid cells have the ability to survive in HAT medium since they have acquired the ability to make HGPRT from the spleen cells. The antibody produced by such a hybrid cell clone is monoclonal (see Figure 3).

Since the beginning of monoclonal antibody technology in 1975, several modifications have been introduced aiming at optimising the product of this useful technique. The first attempt by Köhler and Milstein utilised Sendai virus as the fusing agent (Fusogen). Polyethylene glycol (PEG) has been introduced as a good and convenient fusogen of mammalian cells (Pontecorvo, 1975). Galfre et al (1977) have successfully used PEG to produce mouse hybrids secreting monoclonal antibodies of choice. The addition of dimethyl sulfoxide (DMSO) to PEG solution was shown to enhance the PEG-mediated cell fusion (Norwood et



Figure 3 Schematic representation of the selection of monoclonal antibody producing-hybrids

Mixed Antigen



al, 1976). A large number of chemicals as well as different grades of PEG have been tried to promote fusion events, but low molecular weight PEG (1000-4000) proved to be superior in producing high number of growing somatic cell hybrids (Klebe & Mancuso, 1981).

Pardue et al (1983) have reported a successful in vitro immunisation of spleen cells using recently developed techniques (Luben & Mohler, 1980; Reading, 1982). Mouse spleen cells from an unimmunised animal have been incubated in thymocyte culture conditioned medium with the required antigen. Five days later, these spleen cells have been fused with myeloma cells using PEG solution. This technique is specially useful in situations where very small amounts (nanograms) of antigen are available.

Another useful development has been the introduction of non-secretary myeloma cells. After fusion with these cells, the hybrids secrete only the antibody from B-lymphocytes. The desired monoclonal antibody secretion seems to be amplified (Köhler et al, 1977; Schulman et al, 1978; Kearney et al, 1979).

In addition to mouse-mouse fusions, many attempts have been made to produce hybridomas by rat-rat fusions (Galfre et al, 1979), human-human fusions (Cote et al, 1983; Olsson & Kaplan, 1980; Croce et al, 1980) and mouse-human fusions (Levy & Dilley, 1978; Schlom et al, 1980; Cote et al, 1983). However, permanent cultures of antibody-producing human B-lymphocytes have also been obtained by transformation with Epstein Barr (EBV) virus (Steinitz et al, 1977; Crawford et al, 1983 a & b).

Other developments such as the use of reliable and quick screening assays, including Solid-Phase Radioimmunoassay, ELISA, Fluorescence Microscopy and Fluorescence-Activated Cell Sorting (FACS) techniques have led to the stream lining of monoclonal antibody technology (for more detailed information regarding the use of these

assays, see Current Topics in Microbiology & Immunology (1978) vol. 81, ed. Melchers, Potter & Warner).

#### 1.5.2 Properties and Limitations of Monoclonal Antibodies

The main advantages of monoclonal antibodies over conventional sera lie in the fact that antibodies of chosen specificity and affinity may be produced indefinitely in large and standard amounts. This has major implications for diagnostic, therapeutic and preparative applied research and also makes them a powerful tool in basic research.

##### 1) Diagnostic Applications

Undoubtedly the main advantage of MCAs in diagnosis lies in the fact that large amounts of antibody of the chosen specificity may be produced indefinitely. Many clinical assays have in the past relied on rabbit sera which are variable in titre, specificity, bleed and animal. Standardisation of any particular assay is now possible.

The high specificity gives the advantage of fine diagnosis between two similar strains of the same virus or bacterium or two similar polypeptide hormones. The antibodies may also be used to find tumour associated antigens and to dissect out the aberrant immune response in autoimmune diseases.

##### 2) Therapeutic Applications

Monoclonal antibodies have considerable potential in the field of tumour therapy and have already been used for this purpose. Their high specificity makes it possible, in principle, for antibodies to tumour-associated antigens to be targetted to tumour cells leaving normal tissues unaffected. The antibody can either be used by itself, with reliance on the complement system to potentiate the immune response, or associated with drugs or toxins.

While MCAs may also have potential in the treatment of autoimmune disease via anti-idiotypic therapy or specific suppression of the immune response, this field is at an early stage of development.



### 3) Preparative Applications

The most successful preparative use to date of MCAs has been in the purification of alpha-interferon (Secher & Burke, 1980). The major advantages of monoclonal antibodies in this area lie in that the material used for immunisation need not be pure, though the assay must be specific. It is possible, at least in theory, to isolate a pure component from a very heterogeneous mixture. In practice the response is dominated by major Ags and some degree of prepurification is highly desirable.

The ability to select affinity of Ab is also occasionally an advantage in preparative techniques. Thus it is possible to select an antibody of comparatively low affinity (Mason & Williams, 1980) for affinity purification of a readily denatured antigen.

### 4) Applications in Basic Research

The ability of MCAs to react with a single epitope has extensive implications in many fields of basic research. It is, at least in theory, possible to contemplate a detailed analysis of the distribution within and among cell types of any chosen Ag, to investigate its detailed function by inhibition with Abs directed to different epitopes, to purify it in substantial quantities for detailed structural analysis and potentially to isolate the genes coding for its production. In this last point it has been suggested that the DNA coding for the antibody may even be altered so that an Ab of chosen specificity unobtainable from eukaryotic systems may be produced in bacteria.

The work described in this thesis was directed towards some aspects of these basic applications with chromosomal proteins.

While in theory MCAs have the potential to perform many of the functions outlined above, they have limitations relating to their monoclonal nature. Amongst these limitations are firstly, their over-specificity, for example they may not always react with all strains

of virus. Secondly they may also exhibit under-specificity due to their reaction with single and possibly common epitope such as a carbohydrate moiety. A third disadvantage may be that it has not yet proved possible in practice to employ monoclonal antibodies to dissect a complex array of antigen. Fourthly, functions dependent on the binding of two Abs in close proximity like C'F may not always perform. More detailed accounts of the properties and limitations of monoclonal antibodies are given in recent reviews (Edwards, 1981; Goding, 1980; Staines & Lew, 1980, Yelton & Scharff, 1981; Fazekas de St. Groth & Scheidegger, 1980).

### 1.5.3 Monoclonal Antibodies to Chromosomal Proteins

There is considerable interest in the specific association of certain nonhistone chromosomal proteins with actively transcribed DNA and the possible role of these proteins in chromosome structure and gene expression. The complex and heterogeneous nature of this group means that MCAs have great potential to play a major role in dissecting and characterising such proteins. The use of immunofluorescent methods has helped to locate some such antigens on chromosomes. Saumweber et al (1980) have used the indirect immunofluorescence technique to demonstrate that amongst a number of MCAs raised against the total nuclear fraction from Kc cells of D.melanogaster, fifty eight have shown a highly selective staining pattern on polytene chromosomes of the salivary glands. Three distinct staining patterns have been recognised. Staining, of the transcriptionally active regions, of the phase dark bands and of most interbands, have been noted clearly. The molecular weights of the individual proteins that react with the monoclonal antibodies have been determined. These antibodies have shown reactions with wide range of proteins of different molecular weights. In another work, Howard et al (1981) have produced a monoclonal antibody that recognises a single nonhistone protein of 62K molecular weight in Drosophila. The antibody preferentially stains the induced heat shock



loci. This study demonstrates that MCAs can be used successfully to verify the hypothesis that specific individual NHC proteins are preferentially associated with the set of loci that includes both active sites and those scheduled to be active at some time during development. Among other MCAs produced by Saumweber et al (1980), one has been used in further investigation. Hügle et al (1982) have applied this antibody (T7), which is known to react with transcriptionally active regions, to stain HeLa and PTK2 cells. Using T7; positive immunofluorescent staining on interphase nuclei of HeLa and PTK2 cells has been noted. In an attempt to localise the antigen for this MCA, three polypeptides of molecular weights (44K, 63K and 70K) have been identified. It has been concluded that these polypeptides are probably components of two different RNP structures. One of these structures is sensitive and the other is resistant to RNase treatment. Also among the monoclonal antibodies reported by Saumweber et al (1980), are many whose antigens have been found associated with pulse labelled-RNA (Risau et al, 1982). The distribution pattern of these antigens has been determined in comparison with that of RNA polymerase B by double labelling immunofluorescence using as a reference a MCA directed towards subunit one of Drosophila RNA polymerase B. The results have indicated the existence of two types of RNA-associated particles (Kabisch & Boutz, 1983). This Ab can prove very useful in locating those NHC proteins which have been suggested to play an important role in gene activation and are associated with RNA forming particles (Yang et al, 1981).

Another group of MCAs directed against a nuclear protein of 53K (P53) have been reported. MCAs to (P53) produced by Crawford et al (1981) have shown selective reactivity with human cell lines derived from spontaneous tumours or transformed in vitro with simian virus 40. In contrast, this phosphoprotein has not been detected in normal human cells. Other groups of workers have also described the production of



MCA against a 53K transformation-related nuclear protein (De Leo et al, 1979; Dippold et al, 1981). Dippold et al (1981) have noted that expression of (P53) in human cells correlates with the growth level. High levels of P53 have been found associated with rapid cell proliferation. However, recently Bosslet et al (1983) have described another murine MCA reactive with cell nuclei which is not exclusively against DNA nor P53 in transformed cells but towards a nucleo-protein complex.

Another group of MCAs have been produced against avian red cell nuclear proteins (Kane et al, 1982). Although some of them have shown cross-reactivity with a number of polypeptides which may reflect a common protein domain, the others provided an interesting specificity for the identification and isolation of tissue- and species-specific nuclear components. In more recent studies, Vanderbilt and Anderson (1983) have been able to produce MCAs from mice immunised with erythrocyte chromatin. Tissue specificity has been noted in the immunoreactivity of a number of these Abs. Three protein antigens have been localised as nuclear while another three proteins are found in the cytoplasm. On the other hand another group of MCAs derived from mice injected with chromatin from hen oviduct, react strongly with hen oviduct chromatin (Vanderbilt & Anderson, 1982). All of these Abs react with chromosomal proteins in the absence of nuclear DNA while a large fraction of them reacts specifically with oviduct chromatin but not with other tissues. Another MCA against NHC protein has been reported recently (Kuo et al, 1982).

Teng et al (1982) have raised a highly specific anti HMG-17 MCA. No cross-reactivity with even the closely related HMG proteins (-1, -2 and -14) has been detected. Turner (1981) has successfully produced another group of MCAs to chromatin-associated proteins from human liver, rat liver and a human lymphoblastoid cell line. The

subcellular location of target antigens has been examined by immunofluorescence. Three of these Abs have given staining patterns consistent with the in vivo association of the target Ag with chromatin recognising, respectively, the interphase nucleus and metaphase chromosomes, the nuclear periphery, and the mitotic spindle and other microtubule-containing structures. Moreover, another four MCAs have recognised Ags associated with the intermediate filament network possibly due to cytoplasmic contamination.

### Concluding Remarks and Aim of the Project

As is outlined in the introduction, gene expression is a very complex process which involves the participation of many components, including nucleic acids and various types of nuclear protein. Many of these components are present in very small amounts which makes their characterisation and purification, as well as the assignment of any specific role for each one of them, an extremely difficult task with the limited capacity of the available biochemical techniques. Moreover, understanding of the structural assembly of chromosomes in metaphase or interphase nuclei, and the forces responsible for bringing about the fundamental changes in these structures during the cell cycle is limited.

Many nonhistone nuclear proteins which are involved in different aspects of metabolism are still unpurified and uncharacterised. In addition, proteins involved in the control of gene expression of cells are still largely unidentified. Consequently, highly specific antibodies may be a useful tool in the characterisation of individual chromosomal components.

Specific antibodies could be employed in the following ways:

- (1) In basic research, specific antibodies to minor proteins or proteins that share common biochemical features which make them inseparable or very difficult to purify, could be used to characterise and purify them.
- (2) While cell surface antigens are more commonly used as markers to classify and type cells in culture there are many situations in which the cell surface antigens are not readily resolved. Chromosomal antigens may provide better antigenic resolution in such cases.
- (3) Monospecific antibodies to proteins associated with the control of gene expression could obviously be of great value in the elucidation of this complex process.



- (4) Highly specific antibodies against tumour or other disease-associated antigens could play a valuable role in diagnosis and possibly in treatment.

Consequently, the research undertaken and presented in this thesis is aimed at producing specific antisera and monoclonal antibodies to chromosomal components at the basic level of research but with the anticipation of possible long term clinical significance.

## **Materials & Standard Solutions**

## 2. Materials and Standard Solutions

### 2.1 Materials

The materials used during the course of this project are listed below with their suppliers names and addresses.

#### 2.1.1 Cell Culture Materials

Aminopterin	)	Flow Laboratories, Irvine,
RPMI-1640 medium	)	Scotland
Foetal Calf Serum (FCS)	)	
Penicillin/Streptomycin		Glaxo Pharmaceutical, London
Gentamycin	)	Sigma Chemical Co., Dorset
Hypoxathine	)	England
Thymidine	)	
Non Essential amino acids (NEAA)	)	Gibco Europe, Paisley,
Fungizone	)	Scotland
GMEM medium	)	

#### 2.1.2 Disposable Plasticware

- (1) All tissue culture plasticware and disposable plastic bottles were supplied by Sterilin, Teddington, England
- (2) Costar plates (24-well and 96-well) were supplied by Costar-Inc., Northumbria Biological Ltd., England
- (3) ELISA plates were obtained from Dynatech Laboratories Ltd., Sussex, England
- (4) Flexible microtitre plates for radioimmunoassay were supplied by Flow Laboratories, Scotland

#### 2.1.3 Radiochemicals

L-[<sup>35</sup>S]Methionine, specific activity 500Ci/mmol and [<sup>125</sup>I]iodine (carrier-free Na<sup>125</sup>I), 1.9-2.6Ci/mmol were supplied by the Radiochemical Centre, Amersham, England.



#### 2.1.4 X-ray and Photographic Materials

X-ray films (Kodak X-Omat S) were supplied by Kodak-Pathe-France  
400ASA Photographic films (Kodak) were purchased from a  
photographic retailer, Glasgow, Scotland.

#### 2.1.5 Enzymes

Staphylococcal nuclease (EC 3.1.4.7) was purchased from BCL,  
Bell Lane, Lewes, East Sussex, England.

Ribonuclease A (EC 3.1.4.22); Bovine Pancrease, and DNase I (EC  
3.1.21.1) were obtained from Sigma Chemical Co., Dorset, England.

Trypsin 1:250 (EC 3.4.21.4); Difco, was supplied by Difco  
Laboratories, PO Box 14B, Central Av., East Molesey, Surrey.

#### 2.1.6 Chemicals

The following chemicals were supplied by Sigma Chemical Co.,  
England.

O'-phenyldiamine (OPD), 8-azaguanine (AG), digitonin,  
deoxycholic acid, polyoxyethylene sorbiton monopalmitate 20 (Tween 20),  
N,N,N'-tetramethylene diamine (TEMED), tris (hydroxymethyl) aminoethane  
(Trizma Base), N-desacetyl-N-methyl colchicine (Democolcine),  
O'-dianisidine, spermine, spermidine, N-2-hydroxyethylpiperazine  
N'-2-ethane sulfonic acid (HEPES), Coomassie Blue (G-250).

Other chemicals were supplied as listed below:

dimethyl sulfoxide (DMSO)	)	
2-mercaptoethanol	)	Koch Light Laboratories,
dithiothreitol (DTT)	)	Bucks, England
NaCl and KCl	)	
2,5-diphenyloxazole (PPO)	)	

Polyethylene glycol 1500 (PEG 1500) Serva, Heidelberg, Germany

Triton X-100

Rohm & Haas, Tyne & Wear,  
England.

Pristane (2,6,10,14-tetra-  
methyl pentadecane)

Aldrich Chemical Co.Ltd.,  
Gillingham, Dorset, England

PMSF (phenyl methyl sulfonyl  
fluoride)

Boehringer, West Germany

All other reagents were "Analar" grade and were supplied by  
British Drug House (BDH), Dorset, England.

### 2.1.7 Cell Lines

#### 2.1.7.1 Human Cell Lines

The sources and characteristics of human cell lines which were  
used during the course of this research are listed in Table 2a.

#### 2.1.7.2 Animal Cell Lines

The sources and characters of mouse and other nonhuman cell  
lines used during the course of this research are shown in Table 2b.

### 2.1.8 Animals

#### 2.1.8.1 Mice

BALB/c mice were obtained from the University of Glasgow Colony,  
Animal House, Department of Biochemistry. Mice were propagated by  
sibling mating and entered the study at 2-4 months of age. Both sexes  
were used.

#### 2.1.8.2 Rabbits

White Albino, English, Dutch  $F_3X-F_2Y$  and Half Lop rabbits of  
both sexes entered the study at approximately 8-10 months of age.

### 2.1.9 Serological Materials

Commercial rabbit anti-class specific (mouse) immunoglobulin  
antisera were obtained from Bionetics Laboratory Products, supplied by  
Uniscience, Ltd., Cambridge, England.

Table 2a

Human Cell Lines

<u>Cell Line</u>	<u>Growth Medium</u>	<u>Type/Tissue</u>	<u>Source</u>	<u>Reference</u>
1. Chang liver	GMEM + 10%CS	epithelial (liver)	Gibco-Europe, Scotland	Chang (1954)
2. FL	GMEM + 10%CS + 1% NEAA	epithelial (amnion)	" "	Petursson & Fogh (1963)
3. HeLa	GMEM + 10%CS	epithelial (cervical carcinoma)	Wellcome Tissue Culture Unit, Biochemistry/ Glasgow University	Gey et al (1952)
4. H.Ep-2	GMEM + 10%CS	epidermoid (laryngeal carcinoma)	Dr.J.Pitts Beatson Instit.Glasgow	Moore et al (1955)
5. HT1080	GMEM + 10%CS	firbosarcoma	Dr.R.L.P.Adams Biochemistry/The University	Rasheed et al (1974)
6. K562	RPMI + 10%FCS	Myelogenous Leukemia	Dr.B.Young Beatson Instit.Glasgow	Lozzio & Lozzio (1975)
7. KB	GMEM + 10%CS + 10% NEAA	epithelial (oral carcinoma)	Gibco-Europe, Scotland	Eagle (1955)



Table 2b

Non-human Cell Lines

<u>Cell Line</u>	<u>Growth Medium</u>	<u>Type/Origin</u>	<u>Source</u>	<u>Reference</u>
1. BHK-21	GMEM + 10%CS + 1% NEAA	fibroblast (baby hamster kidney)	Dr.C.McDonald, Instit.Genetics, Glasgow	Ambrose & Ellison (1968)
2. BSC-1	GMEM + 10%CS	epithelial (monkey kidney)	Tissue Culture Unit, Biochemisty/The University	Hopps <u>et al</u> (1963)
3. CHO	GMEM + 10%CS + proline	epithelial (Chinese hamster ovary)	Dr.C.McDonald Genetics/Glasgow	Puck <u>et al</u> (1958)
4. L929	GMEM + 10%CS	connective tissue (mouse)	Tissue Culture Unit, Biochemistry/The University	Sanford <u>et al</u> (1948)
5. P3 (P3-X63-Ag8)	RPMI + 10%FCS	myeloma (BALB/c mouse)	Flow Labs, Irvine, Scotland	Köhler & Milstein (1975)
6. 653 (X63-Ag8-653)	RPMI + 10%FCS	myeloma (BALB/c mouse)	Flow Labs., Irvine, Scotland.	Kearney <u>et al</u> (1979)

Peroxidase conjugated goat anti-rabbit and rabbit anti-mouse were from Miles Laboratories Ltd., Slough, England.

Affinity purified sheep anti-rabbit IgG and anti-mouse IgG were supplied by Dynatech Laboratories, Sussex, England.

Sheep anti-mouse (anti- $\gamma$ ), donkey anti-sheep and other normal sera (rabbit, sheep and goat) were obtained from Scottish Antibody Production Unit (SAPU), Wishaw, Scotland.

Rabbit haemolysin was purchased from Flow Laboratories, Irvine, Scotland.

Guinea pig complement was obtained from Wellcome Diagnostics, Dartford, England.

#### 2.1.10 Sheep Red Blood Cells (SRBCs)

SRBCs were supplied by Professor Douglas of the Veterinary School, University of Glasgow.

#### 2.1.11 Miscellaneous

Ficoll, polyvinylpyrrolidone (PVP), bovine serum albumin (BSA), Protein A and NP-40 were supplied by Sigma Chemical Co., England.

Nitrocellulose papers were purchased from Schleicher & Schüll, West Germany.

Low molecular weight marker proteins and Sephadex G25 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Eppendorf tubes (1.5ml capacity) were obtained from Eppendorf Gerätebau, Hamburg, West Germany.

### 2.2 Standard Solution and Buffers

#### 2.2.1 Fusion and Cell Culture Media

##### 2.2.1.1 RPMI-1640 medium

This was supplied by Flow Laboratories as a dry powder. The content of the bottle was dissolved in distilled water as instructed by the supplier and to it 2g/L of Sodium bicarbonate was added. The pH was adjusted to pH 7.0-7.1 with concentrated HCl and water added up to the

required volume. The medium was sterilised by filtration and then aliquoted and stored at 4°C.

The composition of RPMI medium is shown in Table 3.

Immediately before use, fungizone, penicillin-streptomycin and/or gentamycin and the required amount of heat-inactivated FCS were added.

#### 2.2.1.2 Heat-Inactivation of Foetal Calf Serum (FCS)

Foetal calf serum was inactivated at 56°C for 45-60 minutes, then aliquoted in sterile bottles and stored at -20°C.

#### 2.2.1.3 HT and HAT media

The concentration of HT and HAT in medium is as described by Littlefield (1964). Hundred fold concentrated HT stock was prepared by dissolving hypoxanthine, thymidine and glycine in distilled water. Solubilisation of hypoxanthine was assisted by stirring the mixture at 45-50°C for 60 minutes and the warm solution was sterilised by filtration aliquoted, and stored at -20°C.

HT medium was made up by diluting 1.0ml of the sterilised stock with 100ml of complete FCS-RPMI medium to give a concentration of  $3 \times 10^{-5}$  M,  $1 \times 10^{-3}$  M and  $1.6 \times 10^{-4}$  M for glycine, hypoxanthine and thymidine respectively.

HAT medium was prepared from HT complete medium with the addition, to each 100ml of this medium, 0.4ml of 0.1mM stock aminopterin supplied by Flow Laboratories to give a final concentration of  $4 \times 10^{-7}$  M.

#### 2.2.1.4 GMEM medium

Glasgow modified Eagle's minimum essential medium (GMEM) was supplied by Flow Laboratories. It was made up as 10X concentrated solution and stored at 4°C. This was diluted and supplemented with penicillin/streptomycin, sodium bicarbonate and calf serum (CS) or foetal calf serum (FCS) prior to its use.

The composition of GMEM is given in Table 4.



Table 3

Composition of RPMI 1640 medium

<u>Amino Acids</u>	<u>mg/litre</u>
L-Arginine	200.00
L-Asparagine H <sub>2</sub> O	56.82
L-Aspartic acid	20.00
L-Cystine, disodium salt	59.15
L-Glutamic acid	20.00
L-Gluatmine	300.00
Glycine	10.00
L-Histidine	15.00
L-Hydroxyproline	20.00
L-Isoleucine	50.00
L-Leucine	50.00
L-Lysine HCl	40.00
L-Methionine	15.00
L-Phenylalanine	15.00
L-Proline	20.00
L-Serine	30.00
L-Threonine	20.00
L-Tryptophan	5.00
L-Tyrosine	20.00
L-Valine	20.00
<u>Vitamins</u>	
Biotin	0.20
D-Calcium pantothenate	0.25
Choline chloride	3.00
Folic acid	1.00
i-Inositol	35.00
Nicotinamide	1.00
p-Aminobenzoic acid	1.00
Pyridoxine HCl	1.00
Riboflavin	0.20
Thiamin HCl	1.00
Vitamin B12	0.005
<u>Inorganic salts</u>	
Ca(NO <sub>3</sub> ) <sub>2</sub>	69.49
KCl	400.00
MgSO <sub>4</sub> ·7H <sub>2</sub> O	100.00
NaCl	6000
NaHCO <sub>3</sub>	2000
Na <sub>2</sub> HPO <sub>4</sub>	800.7
<u>Other Compounds</u>	
Glucose	2000
Glutathione	1.00
Sodium phenol red	5.00

Table 4      Composition of Glasgow modified Eagle's Medium (GMEM)

<u>Amino acids</u>	<u>mg/litre</u>
L-Arginine HCl	42.12
L-Cystine disodium salt	28.42
L-Glutamine	584.6
L-Histidine HCl H <sub>2</sub> O	21.00
L-Isoleucine	52.46
L-Leucine	52.46
L-Lysine HCl	73.06
L-Methionine	14.92
L-Phenylalanine	33.02
L-Threonine	47.64
L-Tryptophan	8.16
L-Tyrosine	36.22
L-Valine	46.86
 <u>Vitamins</u>	
D-Ca pantothenate	2.00
Choline chloride	2.00
Folic acid	2.00
i-Inositol	4.00
Nicotinamide	2.00
Pyridaxal HCl	2.00
Riboflavin	0.20
Thiamine HCl	2.00
 <u>Inorganic salts</u>	
CaCl <sub>2</sub> .2H <sub>2</sub> O	264.9
Fe (NO <sub>3</sub> ) <sub>3</sub> .9H <sub>2</sub> O	0.10
KCl	400.00
MgSO <sub>4</sub> .7H <sub>2</sub> O	200.00
NaCl	6400
NaHCO <sub>3</sub>	2750
NaH <sub>2</sub> PO <sub>4</sub> .2H <sub>2</sub> O	140.00
 <u>Other compounds</u>	
Glucose	4500
Sodium phenol red	17.00

#### 2.2.1.5 Polyethylene glycol (PEG) Solution

2.0g of polyethylene glycol 1500 (PEG 1500) was autoclaved at 15 lb/sq.in. for 15 minutes, then cooled down to 45-50°C and added to it 2ml of filter-sterilised 0.15M-HEPES (N-2-hydroxyethyl piperazine N'-2-ethane sulfonic acid) buffer pH 7.5 to give 50% w/v sterile PEG solution.

#### 2.2.1.6 Antibiotics

Penicillin  $10^5$  I.U./L and streptomycin 100mg/L were used in routine culture media from a 100X stock.

For hybridoma production and cloning media, gentamycin (50µg/ml) was used as well as penicillin and streptomycin. Fungizone, 2.5µg/ml in final concentration, was also added.

#### 2.2.1.7 Trypan Blue Dye Solution

This solution was made up by mixing one part of 1% (w/v) trypan blue dye and four parts of 1% NaCl (w/v). The mixture was diluted with saline, to give 0.1% solution, immediately before use for the Trypan Blue Exclusion Test.

### 2.2.2 Chromatin and Dehistonised Chromatin Buffers and Solutions

All solutions and buffers were prepared then stored at -20°C. PMSF was added immediately before use from X100 stock in DMSO.

#### 2.2.2.1 Solution (1)

0.34M	Sucrose
10.0mM	tris-HCl pH 7.5
1.0mM	EDTA
1.0mM	PMSF
0.1%	Triton X-100

#### 2.2.2.2 Solution (2)

80.0mM	NaCl
20.0mM	EDTA
1.0mM	PMSF



2.2.2.3	Solution (3)		
	0.34M	NaCl	
2.2.2.4	Solution (4)		
	7.5M	Urea	
	3.0M	NaCl	
	1.0mM	MgCl <sub>2</sub>	
	0.15M	phosphate buffer pH 6.0	
2.2.2.5	2M Salt buffer		
	2.0M	NaCl	
	10.0mM	tris-HCl pH 6.0	
	1.5mM	MgCl <sub>2</sub>	
	1.0mM	PMSF	
	0.1%	Nonidet-P-40 (NP-40)	
2.2.2.6	Sucrose buffer		
	1.0M	Sucrose	
	2.0M	NaCl	
	10.0mM	tris-HCl pH 6.0	
	1.5mM	MgCl <sub>2</sub>	
	1.0mM	PMSF	
	0.1%	Triton X-100	
2.2.2.7	SSC buffer		
	150.0mM	NaCl	
	15.0mM	Sodium citrate buffer pH 5.5	
<u>2.3</u>	<u>Immunoassay Buffers</u>		
<u>2.3.1</u>	<u>ELISA Buffers</u>		
2.3.1.1	Carbonate/EDTA Buffer (Antigen Coating Buffer)		
	1.41mM	Na <sub>2</sub> CO <sub>3</sub>	)
	1.79mM	NaHCO <sub>3</sub>	) pH 9.6
	1.00mM	EDTA	
	0.02%	NaN <sub>3</sub>	

2.3.1.2 Citrate-phosphate Buffer

113.8mM	$\text{Na}_2\text{HPO}_4$	)
46.45mM	Citric acid	) pH 5.5

2.3.2 Complement Fixation Assay Buffers

2.3.2.1 Stock diluent

0.15M	$\text{MgSO}_4$
0.10M	$\text{CaCl}_2$
1.40M	$\text{NaCl}$
0.10M	tris base

approximately 6.6ml of concentrated HCl added to give a final pH  
7.4 in a total volume of 1L

2.3.2.2 Working diluent (W.D.)

100ml	Stock diluent
1.0g	BSA
Distilled water up to 1 litre	

2.3.3 Saline Buffers

2.3.3.1 Tris-saline (TN)

50.0mM	tris-HCl pH 7.4
150.0mM	$\text{NaCl}$

2.3.3.2 phosphate buffer-saline (PBS)

170.0mM	$\text{NaCl}$
3.4mM	$\text{KCl}$
10.0mM	$\text{Na}_2\text{HPO}_4$ )
1.8mM	$\text{KH}_2\text{PO}_4$ ) pH 7.3

## **Methods**



### 3. METHODS

#### 3.1 Preparation of Nuclei and Chromatin

##### 3.1.1 Preparation of Nuclei

###### 3.1.1.1 The method of Chauveau et al

This was completed after the procedure of Chauveau et al (1956) as modified in "Methods in Enzymology" (1967).

Nuclei were prepared from human cell lines. All steps were done at 4°C.

Cells were washed in PBS and then homogenised in 0.33M sucrose - 4mM CaCl<sub>2</sub>. The homogenate was centrifuged at 600xg for 10 minutes to pellet the nuclei which were resuspended in (0.25M-sucrose, 3mM-CaCl<sub>2</sub>), homogenised and spun at 1500xg for 5 minutes. The pellet was suspended in nine volumes of (2.4M-sucrose, 3mM-CaCl<sub>2</sub>) and homogenised in a tight fitted Teflon pestle in a mechanical homogeniser, then centrifuged at 45,000xg for 1h. The pellet was collected, resuspended in 0.25M sucrose - 3mM CaCl<sub>2</sub>, centrifuged at 600xg for 10 min. The nuclei were collected as a pellet and washed with PBS.

###### 3.1.1.2 Polyamine method

Nuclei were prepared following the method of Blumenthal et al (1979), as modified by Lewis and Laemmli (1982).

###### Solution (I)

7.50mM Tris-HCl pH 7.4

0.10mM Spermine

0.25mM Spermidine

1.00mM EDTA

40.00mM KCl

###### Solution (II)

5.00mM Tris-HCl

0.25mM Spermidine

2.00mM K-EDTA

2.00mM KCl

0.1% Digitonin

pH 7.4

Cells were collected, washed once and soaked at room temperature for 15 min in 100mM-tris-HCl pH 7.4 containing 100mM-NaCl and

5.0mM-MgCl<sub>2</sub>. The mixture was centrifuged at 1400xg for 5 min. The pellet was collected, washed three times at 25°C in 15.0ml of solution (I). The cells were collected after each wash by centrifugation at 1400xg or 5 min. Swollen cells (pellet) were resuspended at 4°C and vortexed for 15 sec in 10.0 ml of twice concentrated solution (I) containing 0.1% digitonin and 10µg/ml of RNAase A. All subsequent steps were carried out at 4°C. The cells were lysed by gentle (5 strokes) and vigorous (5 strokes) homogenisation in a tight fitting pestle. The homogenate was centrifuged at low speed (180xg) for 5 min and the supernatant was collected, layered gently onto 37.0ml of a 15-60% (w/v) sucrose gradient in solution (II) and centrifuged at 4000xg for 5 min then at 1100xg for 30 min.

Cytoskeletal materials remained on top and nuclei were collected as a pellet.

### 3.1.2 Chromatin Preparation

This was carried out after the procedure of Briggs et al (1979) with minor modifications. The solutions used are described in 2.2.2 and all steps were performed at 4°C.

Cells were washed with solution (1) and lysed by gentle homogenisation (1-2 strokes) in 5 volumes of the same solution. The homogenate was spun at 5000xg for 5 minutes. The supernatant referred to as cytoplasmic fraction was stored at -20°C. The pellet was collected, suspended in 3 volumes of solution (2) and homogenised with a Teflon tight-fitting pestle until the nuclei appeared to be broken when examined under the microscope. The mixture was spun at 8000xg for 10 minutes and the pellet was collected, resuspended in 3 volumes of solution (2) lacking PMSF and homogenised again for few strokes. The homogenate was centrifuged at 36,000xg for 15 min. The pellet was extracted by homogenisation in solution (3) and centrifuged at 36,000xg

for 15 minutes. The chromatin was collected as a pellet, resuspended in one volume of 100-fold diluted SSC buffer and pelleted by centrifugation at 36,000xg for 15 minutes.

### 3.1.3 Dehistonised Chromatin

#### 3.1.3.1 2M Salt-extracted chromatin

The chromatin prepared in 3.1.2 or nuclei in 3.1.1 were further extracted by 2M-salt buffer.

The pellet from 3.1.1 and 3.1.2 was resuspended in 3 volumes of 2M-salt buffer described above in 2.2.2.5 and homogenised for several strokes with a tight fitting pestle. Each 2.5ml of the homogenate was layered gently on the top of 2.5ml of sucrose buffer (2.2.2.6) in 5ml capacity cellulose nitrate centrifuge tube. The tubes were spun at 140,000xg in SW50.1 rotor for 90 minutes. The supernatant was decanted gently and the pellet washed twice with double distilled water.

#### 3.1.3.2 2M Salt - 5M urea extracted chromatin

Chromatin preparations as described above in Sections 3.1.2 and 3.1.3.1 were further extracted by 2M salt - 5M urea following the procedure of Briggs et al (1979) with minor modifications.

Chromatin or 2M salt extracted chromatin was suspended in 2mM-tris-HCl pH 7.5, and the concentration of DNA was adjusted to give 0.5mg/ml. Two volumes of solution (4) (2.2.2.4) were added and the solution was stirred for 2h at 4°C then spun at 100,000xg for 24-36h. The supernatant was decanted carefully and the sides of the tubes were rinsed three times with double-distilled water containing 0.1mM-PMSF. The pellet was resuspended in 2mM-tris-HCl pH 7.5, homogenised then stirred at 4°C overnight. The suspension was centrifuged at 1400xg for 5 minutes and the dehistonised chromatin was collected as supernatant.



3.1.4     Bhorjee and Pederson's Method of Preparation of Nucleoli and  
Nonhistone Chromosomal Proteins

This was completed after the procedure of Bhorjee and Pederson (1973). The experiment was performed at 0-4°C.

The cells were washed once with PBS buffer and centrifuged at 500xg for 5 minutes. The pellet was suspended in TNM hypotonic buffer (10mM-tris-HCl pH 7.0, 10mM-NaCl and 1.5mM-MgCl<sub>2</sub>). The suspension was allowed to stand for 10 minutes then homogenised by a tight-fitting Teflon homogeniser (10 strokes). The nuclei were collected by centrifugation at 1000xg for 3 minutes and washed three times in 10 volumes of TNM buffer. The clean nuclei were resuspended at  $4 \times 10^7$  per ml in TNM buffer containing 1mM-PMSF and disrupted by brief sonication (4x15 seconds at a current of 1.5A). The sonicate was then layered on top of equal volume of 30% sucrose bed in TNM buffer and centrifuged at 4500xg for 15 minutes. The nucleoli were collected as a pellet.

The material which remained on top of 30% (w/v) sucrose bed was collected and aliquoted into 10ml portions. Each aliquot was then layered gently on the top of 27ml of 60% sucrose solution (w/v) in TN-EDTA buffer (2.5mM-tris-HCl pH 7.2, 10mM-NaCl & 25mM-EDTA) and centrifuged at 131,000xg for 90 minutes in SW27 Beckman rotor. The pellet was suspended in 0.5-1ml of TNM buffer, and dialysed overnight against several changes of TNM buffer. Histones were extracted from the dialysed chromatin by adding H<sub>2</sub>SO<sub>4</sub> to a concentration of 0.2M and allowing the sample to stand at 4°C for 30 min. The extract was then centrifuged at 37,000xg for 15 minutes. The pellet was re-extracted again with 0.2M-H<sub>2</sub>SO<sub>4</sub> and centrifuged. The nonhistone fraction was pelleted and resuspended in (2.5mM-tris-HCl pH 7.2 and 10mM-NaCl), dialysed against the same buffer overnight.

## 3.2 Animal Procedures

### 3.2.1 Preparation of immunogen in complete Freund's Adjuvent (CFA)

Dehistonised chromatin obtained by procedure 3.1.3 was suspended in PBS. The required amount of immunogen in 50-100 $\mu$ l was then mixed with equal volume of CFA. The mixture was vortexed to make an emulsion which was passed through narrow gauge (G21-G23) needle.

### 3.2.2. Injection of Mice

Individuals were immunised i.p. with 100-150 $\mu$ l of immunogen (measured as 100 $\mu$ g of DNA) as an emulsion in CFA prepared as reported in 3.2.1. The immunisation was repeated twice in CFA with 10-14 days interval in between. A booster of 100 $\mu$ g immunogen in PBS was given i.p. 14-18 days after the second injection. The animals were sacrificed under anaesthetic (ether) 4-5 days after the booster and spleens were collected.

### 3.2.3 Injection Protocol of Rabbits

Individual rabbits were given (70-150 $\mu$ g) of immunogen, (measured as DNA content) emulsified in CFA, subcutaneously (S.C.) in the back of the animal on 2-3 sites. Two S.C. injections were given in CFA two weeks apart, followed by a booster of 100 $\mu$ g of immunogen in PBS 14-20 days after the second injection. The booster was injected i.v. in the ear.

### 3.2.4 Fluid Withdrawal

#### 3.2.4.1 From Mice

##### 3.2.4.1a Blood withdrawal

Blood from mice was withdrawn from the tail 3-4 days after booster. The end of the tail was cut (2mm) by sterile scalpel blade while the animal was under anaesthesia. A few drops of blood could be collected by massaging the tail gently.

##### 3.2.4.1b Ascitic Fluid withdrawal

Ascitic fluids were withdrawn from the intraperitoneal cavity of mice by a syringe attached to 21G gauge needle. The anaesthetised mouse

was laid on the back and carefully a small incision was made through the skin without cutting the peritoneal membrane. A sterile needle (G21) was then inserted and the fluid was withdrawn slowly making sure that the needle did not touch the viscera.

#### 3.2.4.2 From Rabbits

Rabbits were bled from the marginal vein on the ear. The area was cleaned and shaved, then a small cut was made in the vein by a sterile scalpel blade. The blood was drained by massaging and pressing on the vein to stop the return blood flow.

### 3.3 Lymphocyte Hybridoma Production and Tissue Culture

#### 3.3.1 Preparation of Myeloma Cells

Myeloma cells 653 (X63-Ag-653) reported by Kearney et al (1978) were obtained from Flow Laboratories, Scotland.

The cells were grown in RPMI 1640 medium supplemented with 10% FCS, 20µg 8-azaguanine, fungizone, penicillin and streptomycin, at 37°C in humidified 5% CO<sub>2</sub>-incubator. Cells for fusion were collected from culture medium and examined for at least 95% viability.

#### 3.3.2 Preparation of Spleen Cells

Spleen cells as feeder layer and for fusion from immunised BALB/c mice were prepared by the same method. Mice were washed with alcohol and dissected by sterile equipment. Spleens were collected from the mice, washed twice with sterile serum-free RPMI medium containing fungizone and gentamycin as described in 2.2.1.6. The spleen cells were released by teasing the spleen with several injections of RPMI medium. The cells were then washed 3-4 times with RPMI.

#### 3.3.3 Assessment of Cell Viability and Counting

Cell viability was examined by Trypan Blue exclusion test. An aliquot of cell suspension was mixed with an equal volume of trypan blue dye solution (2.2.1.7). The number of live and dead cells were counted in a Neubauer Haemocytometer.

Routine cell counting was also done in a haemocytometer.



#### 3.3.4 Feeder Layer

Spleen feeder cells were prepared as described in 3.3.2. A known number of cells were suspended in 20% FCS-supplemented RPMI medium (with or without HT supplements as described in 3.3.5 and 3.3.6) and dispensed at a concentration of  $10^6$  cells per ml. 0.5 ml of this cell suspension was placed in each well of 2 ml capacity (or 0.1ml in 0.25ml capacity well) 24 hr before fusion or cloning.

#### 3.3.5 Fusion Protocol

This was carried out by the procedure of Pontecorvo (1975) with minor modifications, using HEPES buffer as described by Klebe and Mancuso (1981).

Spleen cells ( $1 \times 10^8$ ) were mixed with ( $1 \times 10^7$ ) myeloma cells in serum-free RPMI medium, washed and centrifuged at 500xg for 5 min. The supernatant was carefully and fully aspirated, and the pellet was tapped gently. To the pellet, 1ml of sterile and pre-warmed ( $37^\circ$ ) 50% (w/v) PEG solution (2.2.1.5) was added slowly over a period of 60 seconds with very gentle shaking. The mixture was incubated at  $37^\circ\text{C}$  for 60 sec and then diluted slowly with 5ml of warm RPMI medium over a period of 3-5 min with gentle shaking. The mixture was again incubated at  $37^\circ\text{C}$ , and left for 5 min, after which 5ml of warm RPMI medium was added and the suspension was spun at 500xg for 5 min.

The cells were then gently resuspended in RPMI 1640 medium containing 20% (v/v) FCS, hypoxanthine, thymidine and glycine (HT medium). Half a millilitre of this suspension was added to each well of 24-well costar plate (2ml capacity) prelayered with a spleen cell feeder layer  $5 \times 10^5$  cells per well as described in 3.3.4, and the plates were incubated at  $37^\circ\text{C}$  in humidified  $\text{CO}_2$  incubator at a density of  $5 \times 10^5$  myeloma cells/ml. After 6-18 h incubation in HT medium, 0.5ml of HT medium containing three times concentrated aminopterin was added to each well, and the plate was again incubated in the  $\text{CO}_2$  incubator for one

week. The cells were fed on days 8 and 11 by removing 1ml of the medium without disturbing the cells, and then 1ml of fresh HAT medium (2.2.1.3) was added slowly.

### 3.3.6 Cloning by Limited Dilution

Screening for positive wells by ELISA was carried out between day 15 and 18 after fusion as described in 3.4.2. Cells in the wells of interest were cloned (first cloning) by limited dilution in HT medium in a 96-well costar plate prelayered with spleen feeder cells ( $10^6$  cells/well). The limiting dilution was performed at 1 cell per well or less. The plates were examined under the microscope after 5-7 days. Wells containing single foci were marked and tested on day 15-20 after cloning. The positive clones were recloned again (second cloning). The positive clones from the second cloning again either expanded or recloned for 3rd or 4th cycle.

Positive single clones at this stage were considered monoclonal and propagated in FCS-supplemented RPMI.

### 3.3.7 Freezing of Cells

Cells were suspended in 1.0ml aliquots containing  $2 \times 10^6$  cells per ml in RPMI-1640 supplemented with 10% (v/v) FCS, 10% (v/v) DMSO in 1.5ml capacity sterile bottles. The aliquots were frozen at  $-70^{\circ}$  overnight, then transferred to Gas-phase liquid nitrogen freezer.

### 3.3.8 Thawing of cells

Frozen cells were quickly thawed in a  $37^{\circ}\text{C}$  water bath and then layered onto the top of 5.0ml of 50% (v/v) FCS in RPMI in a sterile bottle. The bottle was centrifuged at 300xg for 5 min, and the cells were collected and grown in 30-40% (v/v) FCS-RPMI. Half the medium was changed after 24-48 hr with 20% (v/v) FCS-RPMI and then the cells were fed with 10% (v/v) FCS-RPMI as necessary.

### 3.3.9 Propagation of Monoclonal Antibodies

#### 3.3.9.1 In Vivo

In order to establish the monoclonal hybridoma cell lines as tumours in BALB/c mice, the animals were given a primary injection of 0.4ml of Pristane (2,6,10,14-tetramethyl pentadecane) per mouse intra-peritoneally (i.p.) at least seven days before the cells were injected.  $10^6$ - $10^7$  Myeloma cells were suspended in 50-100 $\mu$ l of PBS and injected intra-peritoneally (i.p.) into each mouse. The animals were examined daily after 14 days for the development of Ascites tumour. Ascitic fluid was drained as described in Section 3.2.4.1b

#### 3.3.9.2 In vitro

Hybridoma (or Myeloma) cells were grown in complete RPMI-10% (v/v) FCS medium in sterile flasks at 37°C in a humidified CO<sub>2</sub>-incubator and were subcultured when required before fully confluent. Supernatant (MCA) was collected and stored at -20°C.

### 3.3.10 Routine Culture

Other cell lines were grown in large scale cultures in 2.5L sterile roller bottles. The bottles containing the cultures were gassed to give 5-7% (v/v) CO<sub>2</sub>, sealed and placed on a mechanical rolling device in a 37°C hot room.

The appropriate culture media for each cell line is shown in Table 2.

### 3.4 Assays of Monoclonal Antibodies and Other Antisera

#### 3.4.1 Radioimmunoassay

Affinity purified sheep antiglobulins were labelled with <sup>125</sup>I using the chloramine T method as described in 3.5.3. Flexible microtitre plates were used and the procedure was carried out in duplicate. Protocol:

- 1) 100 $\mu$ l (100 $\mu$ g/ml) of chromatin in carbonate-EDTA buffer (2.3.1.1), was placed in each well and left overnight at 4°C. The plate was then washed twice with TN buffer.



- 2) 150 $\mu$ l of 5% (v/v) goat serum plus 3% (v/v) BSA in TN buffer (GS-BSA-TN) was added to each well to saturate any remaining non-specific binding sites and the plate was left at room temperature for 1h, then washed with TN buffer,
- 3) 80 $\mu$ l of culture fluid (supernatant) or the required dilution of antisera or ascitic fluid in (GS-BSA-TN) was added to each well. The plates were incubated at 4°C overnight (o.n.), washed 3-4 times with TN buffer.
- 4) 70 $\mu$ l of  $^{125}$ I-anti-gamma-globulin ( $10^6$  c.p.m./ml) in GS-BSA-TN buffer was added to each well and the plate was incubated at room temperature for two hours (or overnight at 4°C), wash 4-5 times in TN buffer. The wells then dried, cut, and counted in an LKB minigamma counter.
- 5) Controls: Antigen, antibody and blank (GS-BSA-TN) controls were run simultaneously on the same plate.

#### 3.4.2. Enzyme Linked Immunosorbent Assay (ELISA)

The ELISA technique which was first outlined by Engvall and Perlman (1971) and then modified for the optimal conditions as described by Engvall (1980) was adopted. The reaction was performed in a specially coated ELISA plate (Dynatech).

The protocol was as described for radioimmunoassay in Section 3.4.1, except after step three; 70 $\mu$ l of peroxidase linked anti-mouse IgG in GS-BSA-TN buffer (1/500) was added and the plate was incubated for 2h at 4°C (or 1h at room temperature) and washed 4-5 times with TN buffer. To the wells, 50 $\mu$ l of o-phenyl-diamine (OPD), as a substrate (0.4mg/ml) in citrate-phosphate buffer (2.3.1.2) containing 0.01% fresh  $H_2O_2$ , was added and the plate was kept in the dark for 10-20 minutes or till the colour developed in the wells. The reaction was terminated by the addition of 100 $\mu$ l of 4M  $H_2SO_4$  and the  $A_{492}$  of the solution was read in a Multiskan (Flow) microtitre reader. Controls as described in 3.4.1 were also prepared on the same plate.

### 3.4.3 Complement Fixation Assay (C'F Assay)

This was completed following the method of Wasserman and Levine (1961) with minor modifications.

#### 3.4.3.1 Preparation of complement (C')

Guinea pig Lyophilised complement (Wellcome) was used. The contents of each bottle were dissolved, as recommended by the supplier in 5ml of chilled distilled water at 0°C, then aliquoted in cold (0°C) Eppendorf tubes and stored at -70°C for a maximum period of three months.

When needed, the aliquots were thawed at 0°C and diluted with chilled working diluent (2.3.2.2) and kept at 0°C for up to 1h. The dilution required was estimated by incubating the dilute complement (0.6ml) with 0.2ml of activated SRBCs (3.4.3.3.) at 37°C with shaking. The maximum dilution of complement which gave full cell lysis within 15-20 mins was selected. The maximum dilution was routinely found 1/500.

#### 3.4.3.2 Preparation of Antibody-Antigen-Complement Mixture (Ab-Ag-C')

All steps were carried out at 0-4°C.

Plastic tubes contained serial dilution of antisera (0.1ml each) in working diluent were set up in a duplicate samples. Chromatin samples as prepared in 3.1.2 were suspended in working diluent to give 100µg of DNA per ml and 0.1ml of this suspension was added to each tube. To this mixture, 0.6ml of 1/500 dilution of complement was added and the mixture was incubated at 4°C for 18-20h.

Antibody control for each dilution, chromatin controls (Ag), working diluent, and complement controls were prepared and incubated at the same time.

#### 3.4.3.3 Activation of Sheep Red Blood Cells (SRBCs)

Fresh sheep red blood cells (supplied routinely every 28 days) were washed 2-3 times with working diluent, centrifuged at 300g for 5 mins. The cells were then suspended in working diluent (approximately

1/100) so that 0.2ml of this suspension when lysed with 0.8ml of water gave an  $A_{413}$  of 0.9-1.1.

To this cell suspension rabbit antish sheep haemolysin was added to a concentration of 1 $\mu$ l/ml of suspension. The mixture was incubated at 37°C for 30 min with occasional shaking, then cooled and used the same day.

#### 3.4.3.4 Determination of the percentage of complement fixed by the Antibody-Antigen Complex

After 18-20h, the tubes in 3.4.3.2 were put on ice and 0.2ml of activated SRBCs was added to each one. The tubes were incubated in a shaking water bath at 37°C and watched for complete cell lysis in complement control tubes.

The incubation was then extended for another 2-3 min, and terminated by placing the tubes on ice-bath. The tubes were spun at 500g for 5 mins at 4°C. The supernatants were collected and read at  $A_{413}$ .

The percentage of Complement Fixation (% C'F) is calculated as follows:

$$\% \text{ C'F} = \frac{A_{413} \text{ Complement Control Complete Lysis} - A_{413} \text{ test sample}}{A_{413} \text{ Complete Lysis}} \times 100$$

#### 3.4.4 Determination of the pH Effect on Monoclonal Antibody in ELISA

This was completed as described in 3.4.2, except that serial dilutions of Ab were added in different pH ranges. A range of pH 3 to pH 12 was selected using the Universal Buffer described by Britton and Welford (1937) added to it NaCl to give saline concentration.

The universal buffer was made 0.02857M with respect to boric acid, citric acid, 5,5'-diethyl barbituric acid, potassium dihydrogen phosphate, buffered with NaOH. To this buffer NaCl was added to give a concentration of 0.14M.



#### 3.4.5 Competition Assay

The competition assay was carried out to allow two (or more) different or homologous antigens to compete with each other for the antibody binding site.

In small volumes (150 $\mu$ l) of TN buffer containing 0.2% BSA, serial dilution of different concentrations of antigen (Ag1) were incubated with fixed amounts of antiserum or monoclonal antibody in small glass tubes at 4°C for 18-24h. The tubes were centrifuged briefly at 500xg for 3 min. The unabsorbed antibody (supernatant) was then collected gently and 50 $\mu$ l from each tube was added to the wells of ELISA plate. The plate was precoated 10.0 $\mu$ g of HeLa chromatin (Ag2) and blocked with GS-BSA-TN buffer as described in Section 3.4.2. The procedure was continued as in Section 3.4.2.

#### 3.4.6 Enzyme Treatment of HeLa Chromatin Reacting with Monoclonal Antibody

The procedure of Turner (1981) was adopted with minor modifications. On an ELISA plate, different concentrations of HeLa chromatin were immobilised as described under Section 3.4.2. The plate was then washed with TN buffer. Enzymes (50 $\mu$ l) were added to the wells in the appropriate concentrations and buffer systems. The wells were incubated at 25°C for 60 min (except for RNAase-A which was incubated at 37°C) in a humid atmosphere. The wells were then washed 5 times with TN buffer and blocked with BSA-GS-TN buffer for 1h at room temperature. The plate was washed twice with TN buffer and a fixed amount of monoclonal antibody was added to each well. The procedure was continued as described under Section 3.4.2.

Enzyme-untreated control was also run simultaneously.

The enzyme concentrations and the appropriate buffers are shown below:

1) DNase-I (EC 3.1.2.1.1)

500µg per ml in:

50mM acetate buffer (pH 5.0)

4.2mM MgCl<sub>2</sub>

1.0mM PMSF

2) RNase-A (EC 3.1.4.22)

200µg per ml in:

10.0mM tris-HCl (pH 7.5)

Boiled for 10 min and cooled down immediately before use.

3) Trypsin (EC 3.4.21.4)

200µg per ml in:

10.0mM tris-HCl (pH7.6)

3.5 Immunological and Immunochemical Methods

3.5.1 Chemical Fractionation of Antibodies

Ammonium sulphate precipitation was carried out using the method outlined in the EMBO Laboratory Manual (1979).

Saturated ammonium sulphate solution was prepared by mixing 75g of solid on a total volume of 100ml of distilled water. The mixture was stirred for 1-2h at room temperature then stored at 4°C.

Culture supernatants (or a known volume of ascitic fluid or antiserum, diluted 4 times with PBS) were placed on ice for 10 min. an equal volume of saturated ammonium sulphate was added dropwise with constant stirring at 0-4°C over a period of several minutes. The mixture was left on ice for 30-60 min and then centrifuged at 5000xg for 10 min. The pellet was collected in a small volume of buffer A (10mM tris-HCl pH 7.8 - 40mM NaCl), and transferred into deionised dialysis tubing. Dialysis was carried out against 100 volumes of buffer B (20mM tris-HCl pH 7.8 - 20mM NaCl). The buffer was changed every 3h and a

total dialysis of at least 9h was allowed, after which the sample was collected and spun at 15000xg for 10 min. The supernatant was collected and its  $A_{280}$  measured.

### 3.5.2 Analysis of Biosynthetically Radiolabelled Immunoglobulin

#### 3.5.2.1 Radiolabelling

Cells for labelling were healthy with a viability range of 95-100%. Hybrid cells  $1.5 \times 10^6$  per millilitre were suspended in methionine free-RPMI medium supplemented with 10% dialysed heat-inactivated FCS. [ $^{35}$ S]-methionine solution was evaporated to dryness using a stream of nitrogen in a fume hood or under vacuum. The dry amino acid was redissolved in 10-20 $\mu$ l of methionine-free RPMI and added to the cell suspension to give a final concentration of 150 $\mu$ Ci/ml. The cells were grown in CO<sub>2</sub>-humid incubator at 37°C. Complete RPMI-10% FCS (50 $\mu$ l) was added to the cell suspension 5h after the start of incubation which was continued for a further 11-19h. The cell suspension was then centrifuged at 500xg for 5 min. The pellet was collected for the analysis of the internally labelled-Ig. The supernatant was either frozen at (-70°C) or used for immunoprecipitation.

Foetal calf serum was dialysed against several changes of PBS at 4°C for 18h.

#### 3.5.2.2 Cell Lysis and Immunoprecipitation

##### 3.5.2.2a Cell lysis

The cell pellet was suspended in 0.4ml of cold TKM (100mM tris-HCl pH 8.0, 100mM KCl and 5mM MgCl<sub>2</sub>) buffer. Triton X-100 (10% in TKM), 50 $\mu$ l, was then added and the mixture was left at 0-4°C (with occasional gentle shaking for 10 min to allow cytoplasmic lysis to occur. The lysate was then centrifuged at 500xg for 10 min to pellet nuclei and debris. To the collected supernatant, 50 $\mu$ l of 10%



deoxycholate, 5% SDS in TKM was added. The cell lysate was used immediately for immunoprecipitation.

#### 3.5.2.2b Immunoprecipitation

Supernatant (250 $\mu$ l) from 3.5.2.1 was added to 250 $\mu$ l of TKM buffer containing 2% triton X-100, 2% deoxycholate and 1% SDS. To 90 $\mu$ l of this mixture (or cell lysate mixture in 3.5.2.2a), 1-2 $\mu$ l of rabbit anti-mouse antiserum was added. The mixture was incubated at 4°C for 30-60 min, then 10 $\mu$ l of goat anti-rabbit antiserum was added and the mixture was incubated overnight at 4°C. Control samples containing normal rabbit serum were also run simultaneously.

After the overnight incubation, 50 $\mu$ l of 0.75M sucrose in TKM containing 1% triton X-100, 1% deoxycholate and 0.5% SDS (TKMD) was added and the mixture was layered gently on top of a 0.6ml of two step sucrose gradient (1M - 0.5M) in TKMD. The gradient was centrifuged at 4000xg for 10 min at 4°C, and the top layer was then aspirated gently leaving the pellet which was washed with TKM and collected again.

#### 3.5.2.3 Polyacrylamide Gel Electrophoresis Analysis

The pellet collected from 3.5.2.2b was dissolved in the appropriate volume of sample buffer described in 3.6.1, boiled for 5 min then cooled and applied to 10% gel. The gel contains 0.8:30 acrylamide mixture and was run at constant current of 15mA, stained and destained as described in Section 3.6.2.

#### 3.5.2.4 Fluorography

This was carried out after the procedure of Bonner and Laskey (1974) with minor modifications.

The destained gel which contains the radiolabelled proteins was soaked in DMSO (5 times the volume of the gel) for 30 min with gentle rocking. This was repeated 3 times with fresh DMSO. The gel was then impregnated (with gentle rocking) in four volumes of scintillation solution of 20% w/w 2,5-diphenyloxazole (PPO) in DMSO for 45-60 min.

After this immersion in scintillator, the gel was washed with several changes (10 volumes) of deionised (or distilled) water over a period of 1h. The gel was then dried under vacuum in a gel dryer (Bio-Rad).

In a dark room, X-ray film (X-Omat) was placed in contact with the dry gel and exposed at  $-70^{\circ}\text{C}$  for 4-18h in a dark wrap. The film was developed and fixed in the dark according to the instruction of the developer supplier.

### 3.5.3 Antibody Radiolabelling

The methods of Greenwood et al (1963) were adopted with minor modifications.

Affinity purified sheep anti- $\gamma$ -globulin (Dynatech) was diluted in 0.5M phosphate buffer pH 7.5 to give a concentration of 200 $\mu\text{g}$  IgG per 200 $\mu\text{l}$ . Sodium iodide ( $^{125}\text{I}$ ), 1mCi (carrier-free) was added followed immediately by 100 $\mu\text{g}$ /100 $\mu\text{l}$  of freshly prepared chloramine T in 0.5M phosphate buffer. The mixture was shaken frequently over a period of 60 sec. The reaction was stopped by the addition of 125 $\mu\text{g}$  of sodium metabisulphite in 100 $\mu\text{l}$  of 0.05M phosphate buffer pH 7.5 and the mixture again left for 60 sec with shaking. Potassium iodide 2mg in 200 $\mu\text{l}$  of 0.05M phosphate buffer was added as a carrier for  $^{125}\text{I}$  ions followed by 400 $\mu\text{l}$  of 0.2% BSA in 0.05M phosphate buffer. The mixture was then passed through 1.5x10cm pre-equilibrated sephadex G25 column and the sample was eluted.

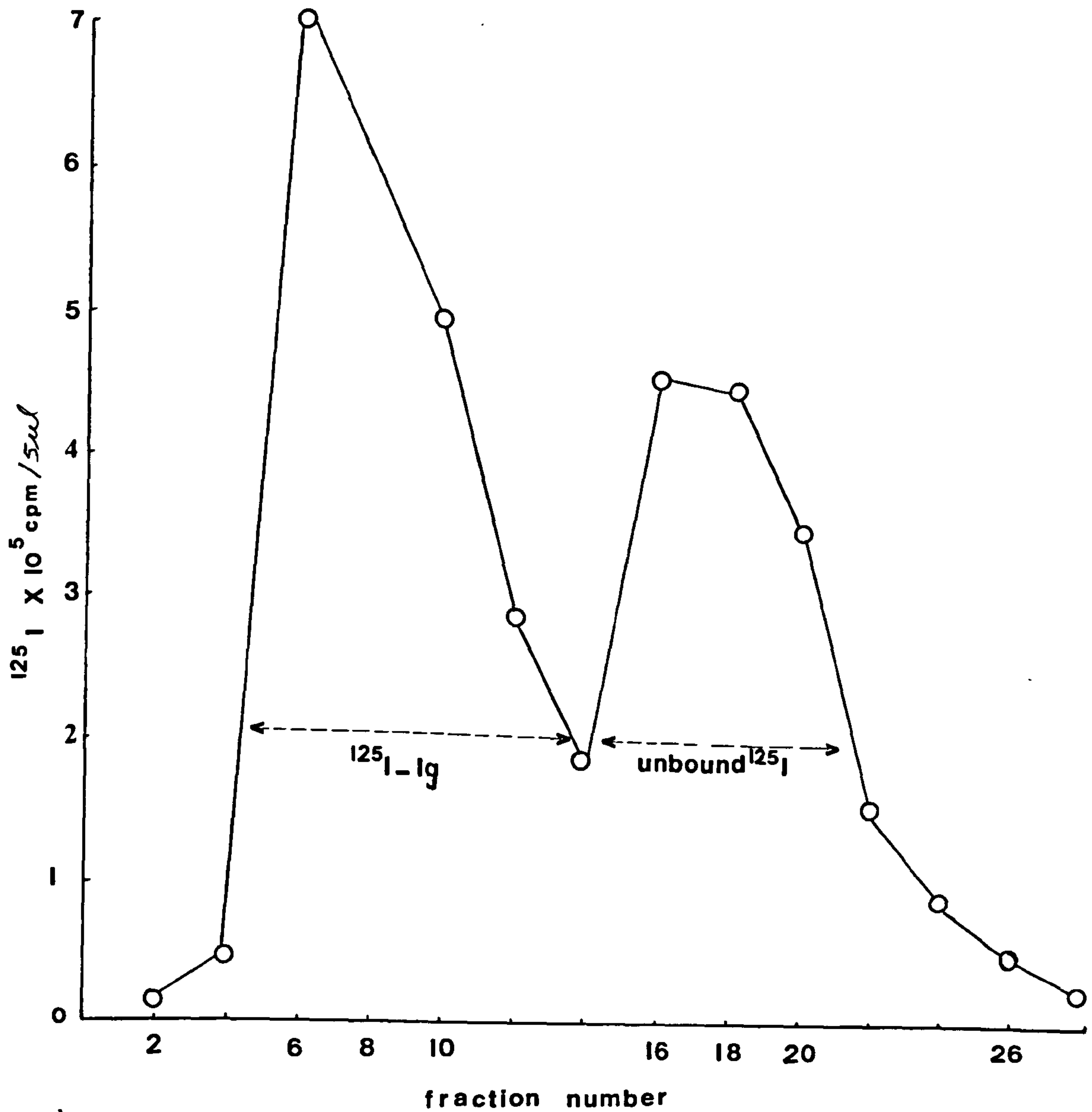
Pre-equilibration and elution were performed by passing 0.1% BSA in 0.05M phosphate buffer through the column. Fractions of 0.5 - 0.7ml were collected.

PMSF was added to the phosphate buffer immediately before use to give a final concentration of 0.1mM.

A typical elution profile is shown in Figure 4.

Figure 4

Elution profile of radiolabelled ( $^{125}\text{I}$ ) antirabbit (or  
antimouse) IgG





### 3.5.4 Immunoblotting

#### 3.5.4.1 ELISA Technique

Antigens (proteins) transferred to nitrocellulose paper (3.6.3) were detected by Enzyme Linked Immunoassay (ELISA) as described by Towbin et al (1979).

Nitrocellulose paper after blotting was washed with TN buffer, and the paper was then incubated with 3% BSA - 5% GS in TN buffer (GS-BSA-TN) for 1h at room temperature to block the nonspecific sites. The paper was washed with TN buffer and incubated with the first antibody (antiserum, ascitic fluid or culture medium) diluted in GS-BSA-TN buffer at 4°C overnight. The paper was then washed 3 times with TN buffer and incubated for 2h at room temperature with peroxidase conjugated second antibody (anti-gamma-globulin) diluted 1/500 - 1/700 in GS-BSA-TN buffer and washed 4-5 times with TN buffer. To visualise the antibody reaction the nitrocellulose paper was incubated in the dark with o-dianisidine 0.0025% w/v ( as a substrate) in TN buffer containing 0.01% H<sub>2</sub>O<sub>2</sub>. The colour developed within 10-20 min and the reaction was stopped by washing the paper in a large volume of TN buffer or deionised water.

#### 3.5.4.2 Immunoblotting by Radiolabelled Antibody

The practical principle was essentially the same as that described in 3.5.4.1 except that the second Ab was <sup>125</sup>I-labelled affinity purified antiglobulin (3.5.3).

The nitrocellulose paper was incubated with the second antibody at 10<sup>6</sup> c.p.m. <sup>125</sup>I per ml of GS-BSA-TN for 2h at 20°C. The paper was then washed 4-5 times with TN buffer, dried by blotting between filter papers and then autoradiographed using Kodak X-ray (X-Omat S) film.

#### 3.5.4.3 Immunoblotting by <sup>125</sup>I-Protein A Method

This was performed after the procedure of Battiger et al (1982) using Tween 20 as blocking agent as modified in our laboratory (A.M.Campbell, personal communication).

The nitrocellulose paper after protein transfer was washed several times in (20mM tris-HCl pH7.2, 0.15M NaCl, 0.5mg/ml NaN<sub>3</sub>, 0.5% Tween 20) for 30-60 min to block non-specific sites. The blot was then incubated (at 4°C overnight) with first antibody (the mouse monoclonal) diluted in the same buffer. The paper was washed 5 times (for 30 min) with the blocking buffer and then incubated (for 4h/at room temperature) again with second antibody (anti-mouse IgM H+L) diluted 1/100 in the same buffer. The nitrocellulose paper was washed again 5 times as described above and then allowed to react with <sup>125</sup>I-protein A (2x10<sup>7</sup> cpm/ml) for 1h, washed (5-10 times over 30-60 min in the blocking buffer), dried by blotting between filter papers then autoradiographed using Kodak X-ray (X-Omat S) film.

### 3.6 Gel Electrophoresis and Protein Blotting

#### 3.6.1 Slab Gel Electrophoresis

This was completed after the method of Laemmli (1970) with minor modifications.

1)	Separating gel buffer	3M tris-HCl pH 8.8
2)	Stacking gel buffer	0.5M tris-HCl pH 6.8
3)	Running (Reservoir) buffer	(0.025M tris base) (0.192M Glycine ) (0.1% SDS ) pH 8.3
4)	Sample buffer	(0.0625M tris-HCl pH 6.8 ) ( 2% SDS ) ( 10% glycerol ) ( 5% 2-mercapto-ethanol) (0.001% bromophenol blue )

5) 30% Acrylamide mixture

a) 0.8:30 (the ratio of bisacrylamide to the total)

29.2g      acrylamide

0.8g      bisacrylamide

**Upto 100ml of distilled water**

The mixture was filtered then stored at 4°C and used only as indicated in the text.

b) 1.2:30

28.8g            acrylamide

1.2g      bisacrylamide

**Upto 100ml of distilled water**

This mixture was used routinely unless otherwise stated in the text.

6) 10% Separating gel mixture 10ml 30% acrylamide mixture

3.75ml separating gel buffer

1.50ml 1% ammonium persulfate

1.67ml 9M urea

15  $\mu$ l      TEMED

upto 30ml Water

This 10% gel mixture was used routinely unless otherwise stated.

7) 3% Stacking gel mixture 2.0ml acrylamide mixture

5.0ml stacking gel buffer

0.5ml ammonium persulfate

**1.13 ml 9M urea**

10  $\mu$ l      TEMED

upto 20ml distilled water

The separating gel mixture was poured between two glass plates (22x30cm) held apart by 1.2mm diameter silicone tubing in addition to 1.1mm-thick spacers on each side. The glass plates were held vertically and the gel allowed to polymerise. The stacking gel was then poured on



the top of the separating gel with a Teflon "comb" in place to provide sample slots. After polymerisation, the sample slots were washed thoroughly with the reservoir buffer to remove the unpolymerised gel solution.

Before loading the samples, the gel was pre-run for 30-60 min at 200V. Samples (15-30µg proteins per 20-50µl of sample buffer) were dissolved in sample buffer, boiled for 3-5 min, cooled and loaded in the sample slots. The gels were normally run at 200V till the dye entered the separating gel, then the run was carried out at constant current of 14-15mA overnight.

The run was terminated when the dye front reached 2cm from the bottom of the gel.

### 3.6.2 Staining and Destaining of Gels

Slab gels were stained with 0.25% w/v coomassie blue R250 (Sigma) in 45:10:45 methanol: acetic acid: water overnight at room temperature or for 1h at 37°C.

Gels were destained in several changes of 45:10:45 methanol: acetic acid: water. The percentage of methanol was decreased gradually up to 20%.

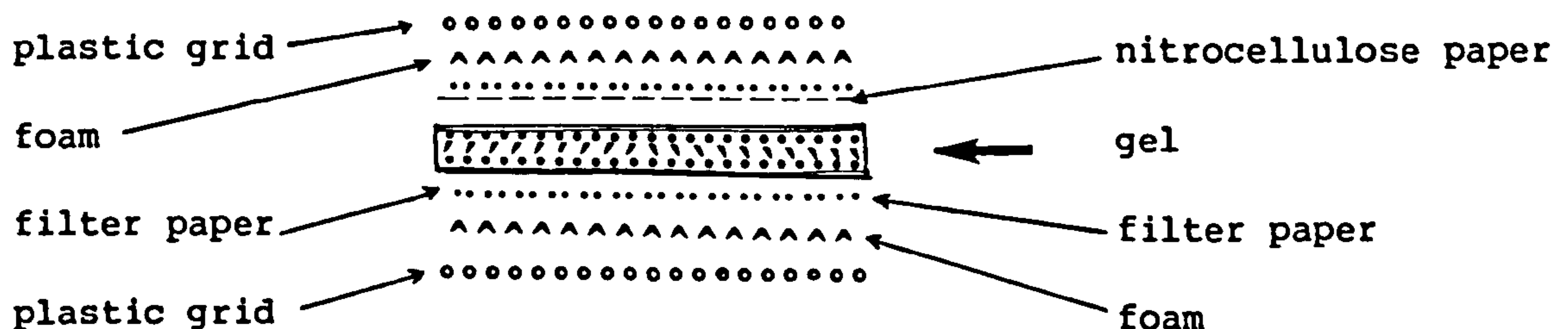
### 3.6.3 Protein Blotting

#### 3.6.3.1 Electroblotting

This was completed after the method of Towbin et al (1979). The assembly of the gel and nitrocellulose paper is illustrated below. Basically, a plastic grid followed by foam pad (Scotch-Brite pad) and then wet filterpaper 3mm (Whatman) were laid flat underneath the slab gel. On the top of the gel, buffer-soaked nitrocellulose paper was laid carefully excluding any air bubble which might be formed between the gel and paper. The paper was followed by wet filter paper and another foam pad and plastic grid. The assembly was tied tightly by rubber bands and

then immersed vertically in an electrophoretic destainer tank containing 20% methanol in 24mM tris-192mM glycine pH 8.3. The nitrocellulose paper was placed facing towards the anode (+ve) and the gel facing the cathode (-ve). The electrophoretic run was at 300mA for 2.5-3h.

The plastic grids contained many holes to allow the current to flow freely.



### 3.6.3.2 Blotting by Diffusion

The procedure of Bowen et al (1980) was adopted.

#### Transfer Buffer

50.0mM NaCl

2.0mM EDTA

0.1mM DTT (fresh)

10.0mM tris-HCl pH 7.0

#### Urea Pretransfer Buffer

4M urea in transfer buffer

Before blotting, the gels were immersed in urea pretransfer buffer and agitated for 3h. The assembly of gel for blotting was basically as described in 3.6.3.1 except that nitrocellulose papers were put on each side of the gel and the transfer was performed by immersing the whole assembly in transfer buffer for 36-48h.

### 3.6.3.3 Staining of Blots

Protein on nitrocellulose paper was stained by 0.1% freshly prepared amidoblack (Naphthalene - blue black - Sigma), made up in 45:10:45 methanol:acetic acid:water, for 10 min.

### 3.6.3.4 Destaining of Blots

Nitrocellulose papers were destained immediately after staining by several changes of 90:2:8 methanol:acetic acid:water.

### 3.6.3.5 DNA Binding on the Blot

The method of Bowen et al (1980) was employed:

	( 10.0mM	tris-HCl pH 7.0
	( 1.0mM	EDTA
	( 0.02%	BSA
DNA-binding buffer	( 0.02%	Ficoll
	( 0.02%	polyvinyl pyrrollidone
	(	(PVP)
	( 50.0mM	NaCl

To determine whether the antigenic determinants of the proteins transferred on nitrocellulose paper are DNA-dependent or not, the proteins on the paper were reconstituted with DNA. After blotting, the paper was washed with DNA binding buffer and incubated with 100µg/ml DNA in the same buffer at 37-40°C for 2h with continuous shaking. The paper was washed with TN buffer and treated as described in 3.5.4.

## 3.7 Immunofluorescence

### 3.7.1 Preparation of Monolayer Cells

Round glass coverslips were washed with dilute chlorox solution, rinsed with distilled water then dried and sterilised in a hot air oven at 400°C. The coverslips were cooled down and 4-5 of them were placed in a sterile 60mm plastic petridish. Cells at  $10^5/50\mu\text{l}$  were placed on the top of coverslips and grown for 48h in the appropriate medium in a CO<sub>2</sub> incubator.

### 3.7.2 Preparation of Mitotic Cells

Metaphase cells were prepared exactly as described in 3.7.1. except that the medium was supplemented with 0.5µg/ml final concentration of Democolcine (N-desacetyl-N-methyl colchicine) 8-15h before collecting the cells.



### 3.7.3. Lysis and Fixation of Cells to Preserve Keratins

<u>Hypotonic buffer</u>		<u>Fluorescence Buffer (F)</u>	
5.0mM	MgCl <sub>2</sub>	130 mM	KCl
2.0mM	EGTA	5.0mM	NaCl
1.0mM	DTT	1.0mM	NaN <sub>3</sub>
5.0mM	NaN <sub>3</sub>	5.0mM	MgCl <sub>2</sub>
0.5mM	PMSF	1.0mM	EGTA
5.0mM	tris-HCl pH 7.5	20.0mM	potassium phosphate buffer pH 7.5

Cells were lysed on coverslips according to the procedure of Granger and Lazarides (1982) with minor modifications.

Cells attached to coverslips were washed with PBS and lysed by immersion in several changes of cold hypotonic buffer at 0-4°C for 10 min. The coverslips then were held face-up position in the bottom of a beaker filled with hypotonic buffer up to a height of 3-4cm. The cells were further disrupted by sonication. The sonicator probe tip was positioned 3-4cm overhead and sonication was at 1.5 - 1.7V for 15-20 sec using 6.0mm diameter Ti probe. Disrupted cells on coverslips were then fixed with 3% gluteraldehyde (Electron Microscopy Grade) in buffer (F) for 30-60 min at room temperature.

After fixation, the cells were made permeable by immersion in cold acetone (-10°C) for 10 min.

### 3.7.4 Cells Preparation on Coverslips for General Immunofluorescence Staining

The procedure of Stenman et al (1975) was adopted. The cells were grown on coverslips as described in section 3.7.1, washed once with cold PBS and treated with ice-cold hypotonic medium (10mM tris-HCl pH 7.4, 40mM glycerol, 20mM NaCl, 5mM KCl, 1mM CaCl<sub>2</sub> and 0.5mM MgCl<sub>2</sub>) for 10 min. The excess of buffer on coverslips was blotted gently and

the coverslips were air-dried. The cells were fixed in ethanol:acetone (1:1) at  $-20^{\circ}\text{C}$  for 10 min, airdried and then treated with antibodies as described under Section 3.7.5 except that the dilutions of antisera were performed in 0.2% BSA in PBS.

#### 3.7.5 Treatment of cells with Antibodies

Acetone treated cells were washed with PBS and incubated for 30-45 min at room temperature with first antibody (antisera or MCA) diluted in TN buffer. The coverslips were washed 3 times with PBS then incubated for 20 min at room temperature with second antibody (goat anti mouse - or rabbit - IgG H+L) coupled to FITC (Miles) diluted 1/40-1/30 in PBS. The coverslips were washed 3-4 times with PBS and mounted with 90% glycerol in (85mM  $\text{NaHCO}_3$  and 15mM  $\text{Na}_2\text{CO}_3$  pH 8.9) and viewed by "Leitz" fluorescent microscope.

#### 3.7.6 Chromosome Spread Preparation

Healthy cells were grown in plastic tissue culture bottles for 48h in the appropriate medium. Democolocine (N-desacetyl-N-methyl colchicine) was added to a final concentration of 5 $\mu\text{g}/\text{ml}$  and the cells were incubated for 45-60 min. The mitotic cells were then harvested by gentle rocking of the plastic bottle and the cells were washed with PBS and centrifuged at 500xg for 5 min. The cells were resuspended in 0.075M KCl and allowed to stand for 10-12 min then centrifuged at 500xg for 5 min. All the KCl solution was sucked off except 0.25-0.5ml. The cells were resuspended very gently in the remainder of KCl solution and then fixed in cold 3:1 methanol:acetic acid fixative mixture by pouring 5ml slowly dropwise and another 5ml quickly. The cells were left in the fixative mixture for at least 30 min (up to 24h) then centrifuged at 500xg for 5 min. The pellet was resuspended in 10ml of fixative mixture and centrifuged again. The collected pellet was resuspended in 1-2ml of fixative mixture. From this suspension 5-6 drops were dropped from a height of 100cm onto a slide (prewashed with chromic acid for at least 2h then dried).

### 3.7.7 Chromosome Staining

Chromosomes which were spread on a slide as described in Section 3.7.6 were either treated with antibodies as in 3.7.4 for immunofluorescent staining or stained with Giemsa stain as follows.

The chromosomes fixed on the slides were stained with Giemsa stain (10%) in Gurr's buffer (BDH) pH 6.8. The staining was performed for 30 min at room temperature and the slides were washed thoroughly under tap water and dried.

### 3.7.8 Photography

Cells or chromosomes were viewed in Fluorescence Microscope (Leitz) using different magnifications or an oil immersion lens with magnification of X2000. Photographs were taken using 400ASA film (Kodak) attached to an Orthomat camera.

## 3.8 Miscellaneous

### 3.8.1 DNA Preparation

The procedure of Flint et al (1976) was adopted with minor modifications.

Cells were washed in solution (1) (2.2.2.1), vortexed and homogenised gently (one stroke), then spun at 500xg for 5 min. The pellet was collected, resuspended in hypotonic buffer (10.0mM tris-HCl, 1.0mM EDTA, 0.5% SDS pH 7.9) and vortexed. The suspension was extracted twice with phenol saturated with hypotonic buffer lacking SDS. The aqueous layer was collected each time and then extracted further with isoamylalcohol:chloroform 1:24 and the aqueous phase collected. Sodium acetate 3M (1/10th volume) was then added to give a final concentration of 0.3M. Cold ethanol (2 volumes) was then added and nucleic acids were precipitated at  $-20^{\circ}$  overnight.



The precipitate was collected, added to 20-25ml of tris-HCl pH 7.9 and incubated with 50µg/ml of ribonuclease A (boiled for 10 min and cooled on ice immediately before use) at 37°C for 60 min. The mixture was then extracted twice with saturated phenol and once with isoamylalcohol-chloroform. Two volumes of cold ethanol was added to the aqueous layer to precipitate DNA at -20°C.

### 3.8.2 DNA Digestion

Chromatin or dehistonised chromatin was suspended in digestion buffer (10mM tris-HCl, 40mM NaCl, 1.0mM CaCl<sub>2</sub>, 1mM PMSF pH 7.5) at a concentration of 0.60mg DNA/ml. Staphyloccocal nuclease (Boehringer) 15,000U in 1ml of digestion buffer was then added and the mixture was vortexed and incubated at 37°C for 90-120 min in a shaking water bath. The reaction was terminated by the addition of EDTA to a final concentration of 2.0mM.

### 3.8.3. DNA Reconstitution

The nuclease digested chromatin was reconstituted in the following manner.

The digested chromatin (100µg) was dialysed against 500ml of (5M urea, 2mM EDTA, 2.5M NaCl and 50mM tris-HCl pH 7.5) at 4°C for 18h. The dialysing buffer was changed four times over a period of 36-48h bringing the concentration of NaCl down to 40mM then urea was dialysed out (3-4 changes) against (40mM NaCl, 1mM EDTA and 50mM tris-HCl pH 7.5) overnight at 4°C.

If the digested chromatin was prepared for C'F assay, further dialysis (2 changes) against working diluent was necessary. To reconstitute native DNA with the digested chromatin, the required amount of DNA (homologous or heterologous) was added to the chromatin before the last change of dialysis.

#### 3.8.4 DNA Estimation

The procedure of Burton (1956) was used with minor modification.

A duplicate series of test tubes were prepared containing 20, 40, 60 and 100 $\mu$ g per 100 $\mu$ l of standard calf DNA solution (from a stock 1mg/ml distilled water).  $\text{HClO}_4$  0.5M solution (0.5ml) was added to each tube and the mixtures were incubated at 70°C for 30 min, cooled to room temperature and centrifuged at 600xg for 10 min. The supernatant was collected, 2.0ml of diphenylamine reagent was added to each supernatant and the solutions were kept in the dark at room temperature for 20-24h. The  $A_{600}$  of the solution was read and plotted on graph paper. A typical straight line was obtained within the range of concentrations used as shown in Figure 5. Any unknown DNA sample was treated in the same way and its concentration was obtained from the standard curve.

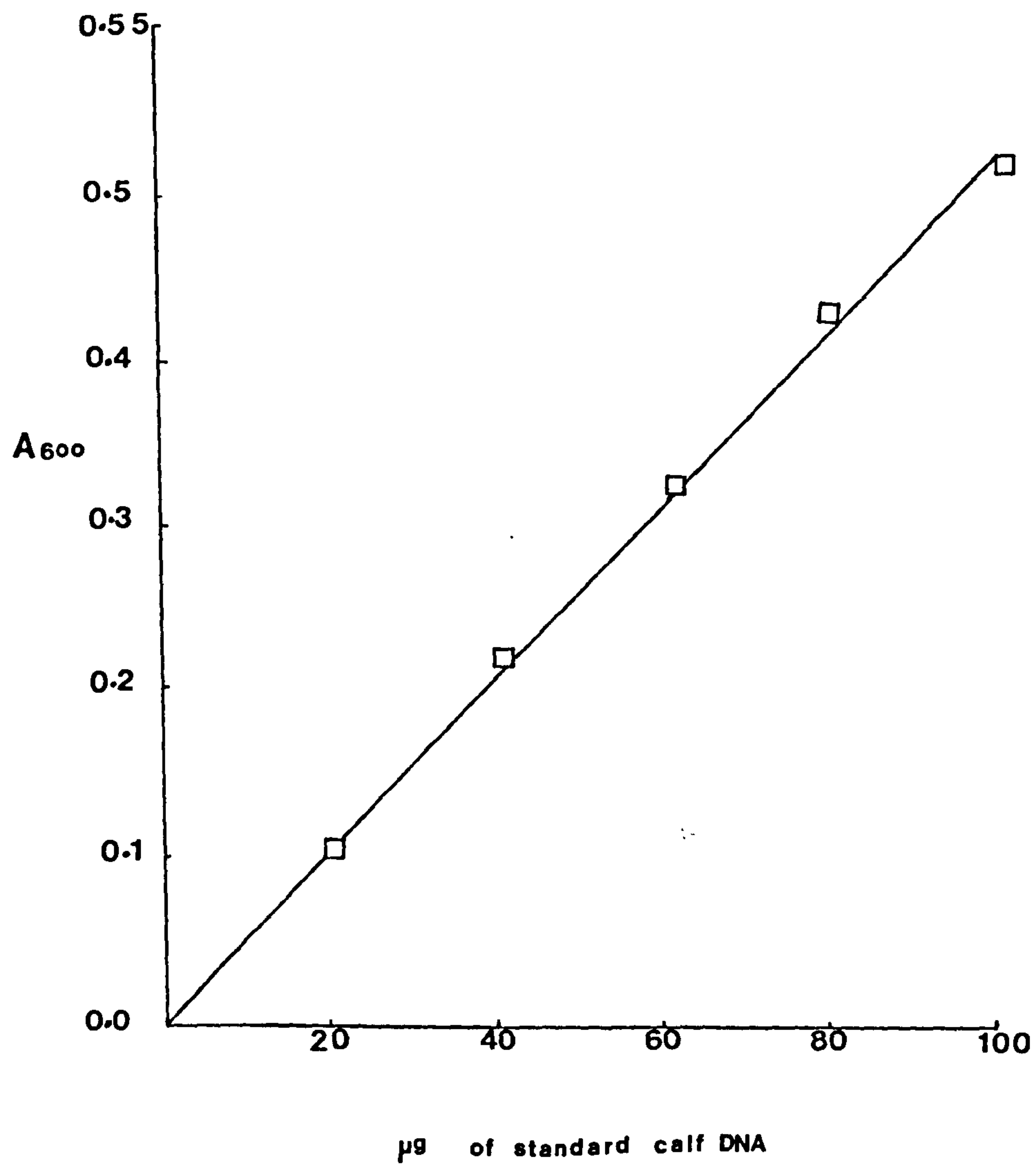
Diphenylamine (DPA) reagent was prepared by dissolving 1.5g of DPA in 100ml of glacial acetic acid added to 1.5ml of concentrated sulphuric acid and the reagent was stored in the dark at room temperature. Before this reagent was used, a 20ml aliquot was withdrawn and 0.1ml of aqueous acetaldehyde (16mg/ml) added to it. For routine measurement of DNA concentration, 1  $A_{260}$  unit was found to be equivalent to 50 $\mu$ g/ml.

#### 3.8.5 Protein Estimation

The procedure of Bradford (1976) was adopted.

A duplicate series of test tubes containing 10, 20, 30, 40 and 50 $\mu$ g/0.1ml of standard BSA solution (from a 1mg/ml stock) were prepared in 150mM NaCl. The volume in the test tubes was kept constant at 0.1ml. Bradford's reagent (3.0ml) was then added and the tubes were vortexed and left to stand for 10 min to complete the reaction. The  $A_{595}$  of the coloured solution obtained was read and a standard curve was drawn.

Figure(5): Standard Curve For DNA Estimation





To estimate an unknown protein sample concentration, the protein was treated exactly in the same way as for the standard and its concentration was obtained from the standard curve. The standard curve is shown in Figure 6.

Bradford's reagent was freshly prepared by dissolving 100mg of Coomassie Brilliant Blue G-250 (Sigma) in 50ml of 95% ethanol. This solution was mixed with 100ml of 85% w/v phosphoric acid, diluted with distilled water to a volume of 1L, and filtered.

#### 3.8.6 Intermediate Filaments Preparation (IF)

This was completed after the procedure of Schmidt et al (1982) with minor modification.

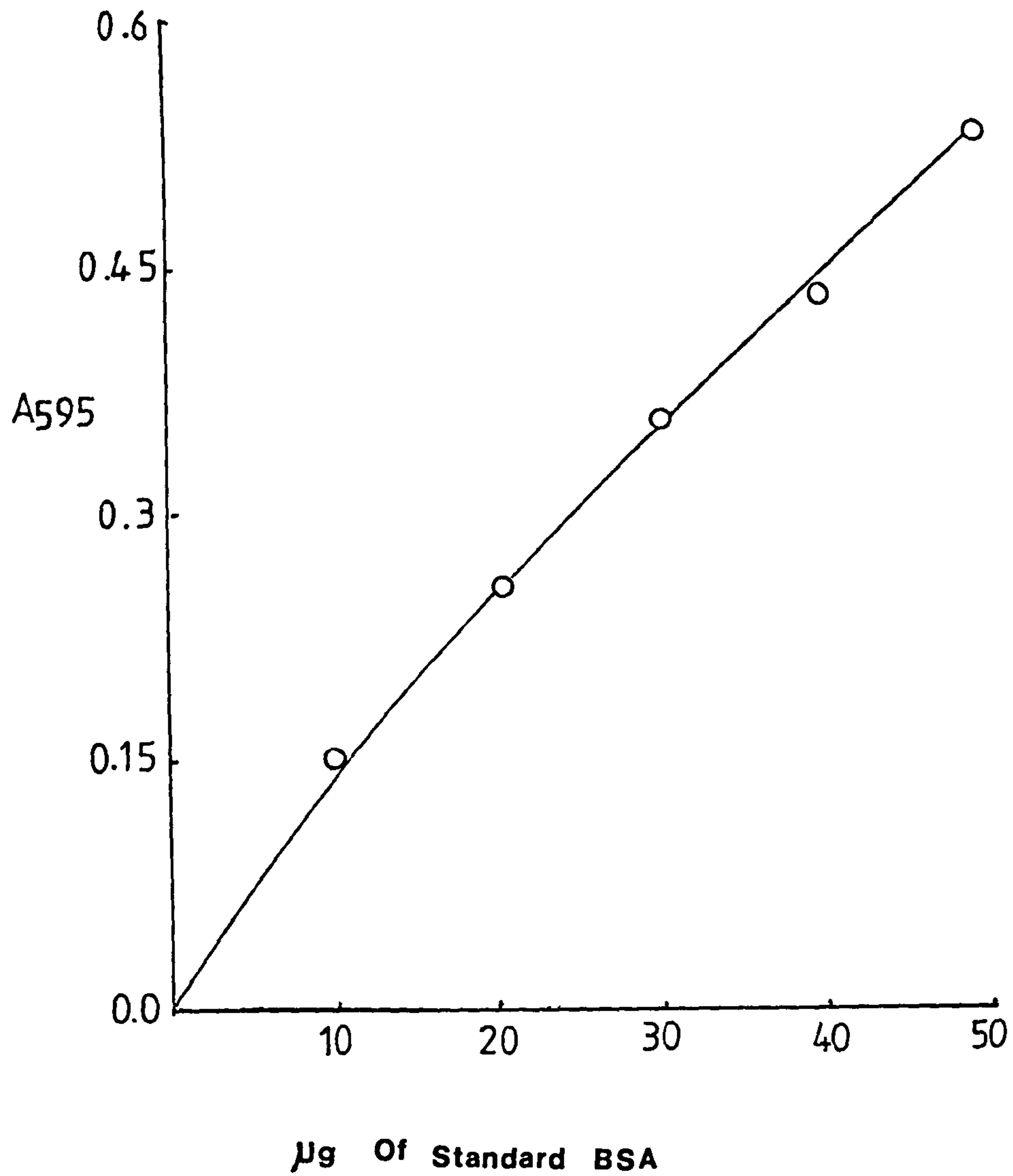
The cells (1-2g) were lysed by homogenisation (15 strokes) in a tight-fitting pestle in lysis buffer (0.3M KCl, 0.15M potassium phosphate buffer pH 6.5). The homogenate was stirred gently for 20 min at 4°C and homogenised again, diluted with 4 volumes of cold distilled water, centrifuged at 800xg for 10 min. The pellet was then suspended in 4 volumes of 0.4% NaHCO<sub>3</sub> at 4°C, stirred gently for 10 min, centrifuged at very low (180xg) speed for 3 min. The pellet was collected, resuspended in 0.05M NaHCO<sub>3</sub> - 0.05M Na<sub>2</sub>CO<sub>3</sub> (1g pellet/ml), stirred for 10 min, diluted with 10 volumes of 0.5mM CaCl<sub>2</sub> and centrifuged at 800xg for 10 min.

To make acetone powder of IF, the pellet was added to three volumes of acetone, mixed and spun at 180xg for 5 min. The residue was collected, mixed with one volume of acetone containing 0.5mM Na<sub>2</sub>CO<sub>3</sub> and air dried.

#### 3.8.7 Nuclear Lamina Protein-Matrix Preparation

The method of McKeon et al. (1983) was followed with minor modifications.

**Figure 6 : Standard Curve For Protein Estimation**



HeLa cells ( $20 \times 10^6$ ) were washed and extracted by four strokes in a tight-fitting pestle (Teflon) in 2.0ml of PBS (section 2.3.3.2) lacking KCl but containing 0.1% Nonidet P-40, 15.0mM 2-mercaptoethanol and 1.0mM PMSF. The mixture was then centrifuged at 1000xg for 10 min and the pellet was resuspended in 2.0ml of the same buffer containing 5.0mM  $\text{CaCl}_2$  and exposed to 10 units/ml of micrococcal nuclease for 10 min at 22°C. The reaction was stopped by the addition of EDTA to a concentration of 3.0mM and the mixture was centrifuged at 1000xg for 10 min. The pellet was extracted by 1.0ml of 3M NaCl (4 strokes in glass homogeniser) in a buffer containing 80mM KCl, 5mM EDTA, 0.1% triton X-100 and 15.0mM Pipes (1,4-piperazinediethane sulfonic acid, pH7.4). The homogenate was then centrifuged at 2000xg/10 min and the pellet was collected.



#### 4. Results and Discussion

#### 4.1                      Results Obtained Using R3.1 Antiserum

##### Results

R3.1 is a polyclonal antiserum obtained after immunising a (Half Lop) rabbit with two subcutaneous injections of histone-depleted HeLa chromatin (150µg of DNA) extracted with 2M salt and 5M urea, prepared as described in Section 3.1.3.2. A booster injection of the antigen (150µg) was given intravenously six days before bleeding of the animal.

The antiserum shows a high titre of immunoreactivity towards HeLa chromatin even at a dilution of 1/1200. This has been shown by C'F assay and ELISA (Figures 4.1.1, 4.1.11 & 4.1.12).

Using ELISA, the antiserum (R3.1) reacted in a similar pattern, when between 2-18µg of HeLa chromatin was layered in each well of the ELISA plate (Figure 4.1.1). If the amount of chromatin plated in each well was reduced to 1µg/well or less, the immunoreactivity of R3.1 fell drastically. This gives an indication of the limited amount of antigen which could be immobilised in each well of the ELISA plate.

The target antigen requires the presence of DNA for its immunoreactivity with R3.1 antiserum. This is demonstrated by the great loss of activity on micrococcal nuclease digestion as seen in ELISA (Figure 4.1.2) and C'F assay (Figure 4.1.3). Since micrococcal nuclease digests both DNA and RNA, it is possible that the complex antigen may require RNA to determine the configuration of the antigenic determinants and to make them accessible for the antibody recognition. The immunoactivity of the complexed antigen was almost completely restored upon reconstituting the nuclease-digested chromatin with HeLa DNA alone (Figures 4.1.2 & 4.1.3). As the HeLa DNA was prepared in a way which eliminates the presence of RNA by digestion with large amounts of RNAase A, as described in Section 3.8.1, DNA rather than RNA is therefore required for the immunoreactivity of R3.1 antiserum with HeLa chromatin. In order to demonstrate whether the immunoreactivity of the

Figure 4.1.1

ELISA of R3.1 Antiserum with HeLa chromatin

Enzyme linked immunosorbent assay of different concentrations of R3.1 antiserum reacted with various amounts of HeLa chromatin layered in the wells of ELISA plate. (Antibody and antigen controls were subtracted).

○	18μg	chromatin
□	2μg	chromatin
Δ	1μg	chromatin
×	10ng	chromatin

The highest antibody control was 0.07 A<sub>492</sub> unit at 1/150 dilution while the highest antigen control was found to be 0.15 A<sub>492</sub> at 18ug of chromatin.



**Figure 4.1.1**

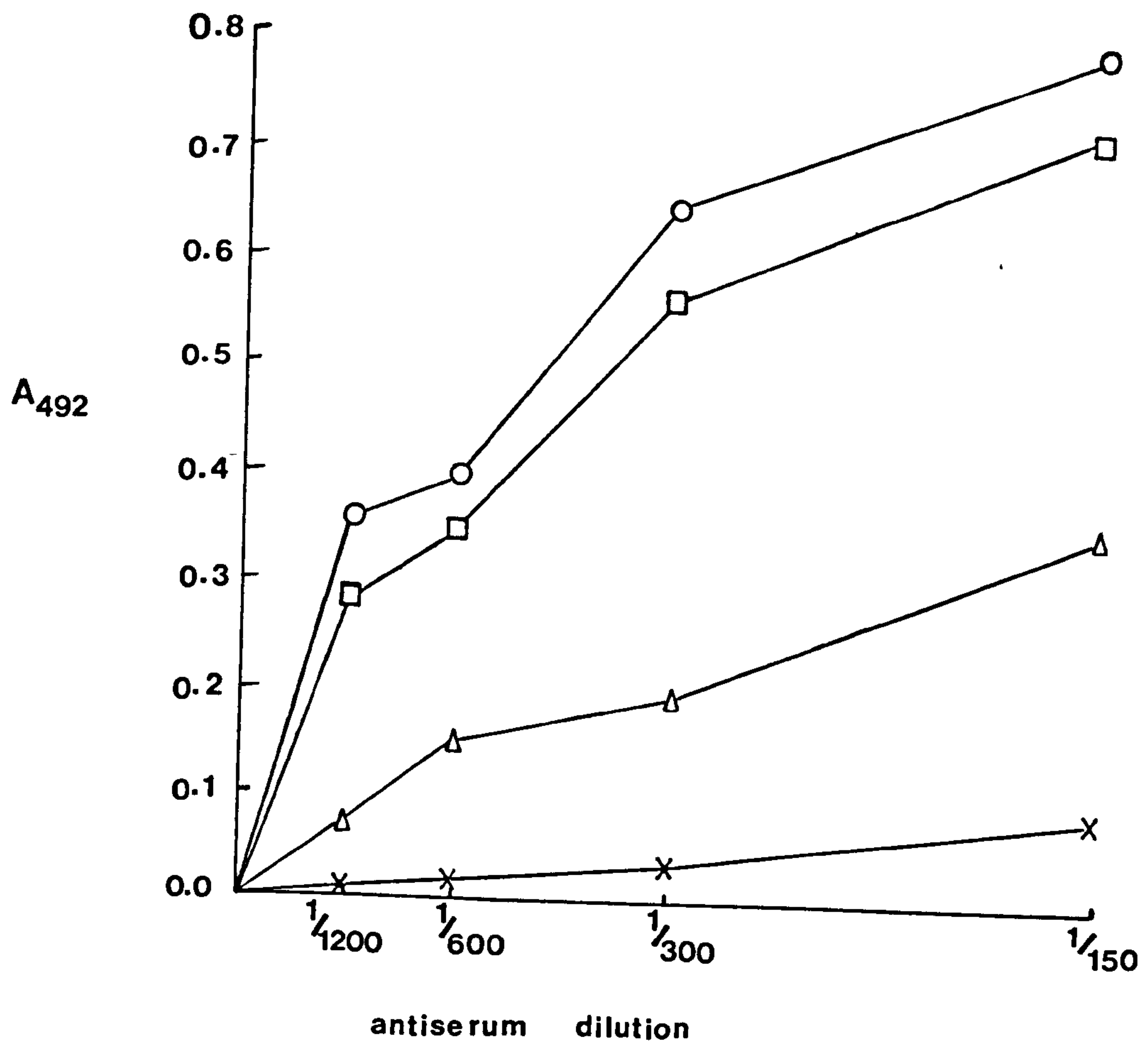


Figure 4.1.2

ELISA of R3.1 Antiserum with HeLa Digested- and  
Undigested-chromatins

Enzyme linked immunosorbent assay of R3.1 antiserum (1/250) allowed to react with different concentrations of HeLa antigen immobilised on an ELISA plate. The Chromatins measured as total protein content and the DNA values estimated as total DNA applied on the ELISA plate. (Antibody and antigen controls have been subtracted).

- Undigested HeLa chromatin
- Micrococcal nuclease-digested HeLa chromatin  
reconstituted with HeLa DNA
- Δ Micrococcal nuclease-digested HeLa chromatin
- ∇ HeLa DNA alone

Antibody control was found to be 0.04 A<sub>492</sub> unit, while antigen controls were all between 0.14-0.1 A<sub>492</sub> unit.

**Figure 4.1.2**

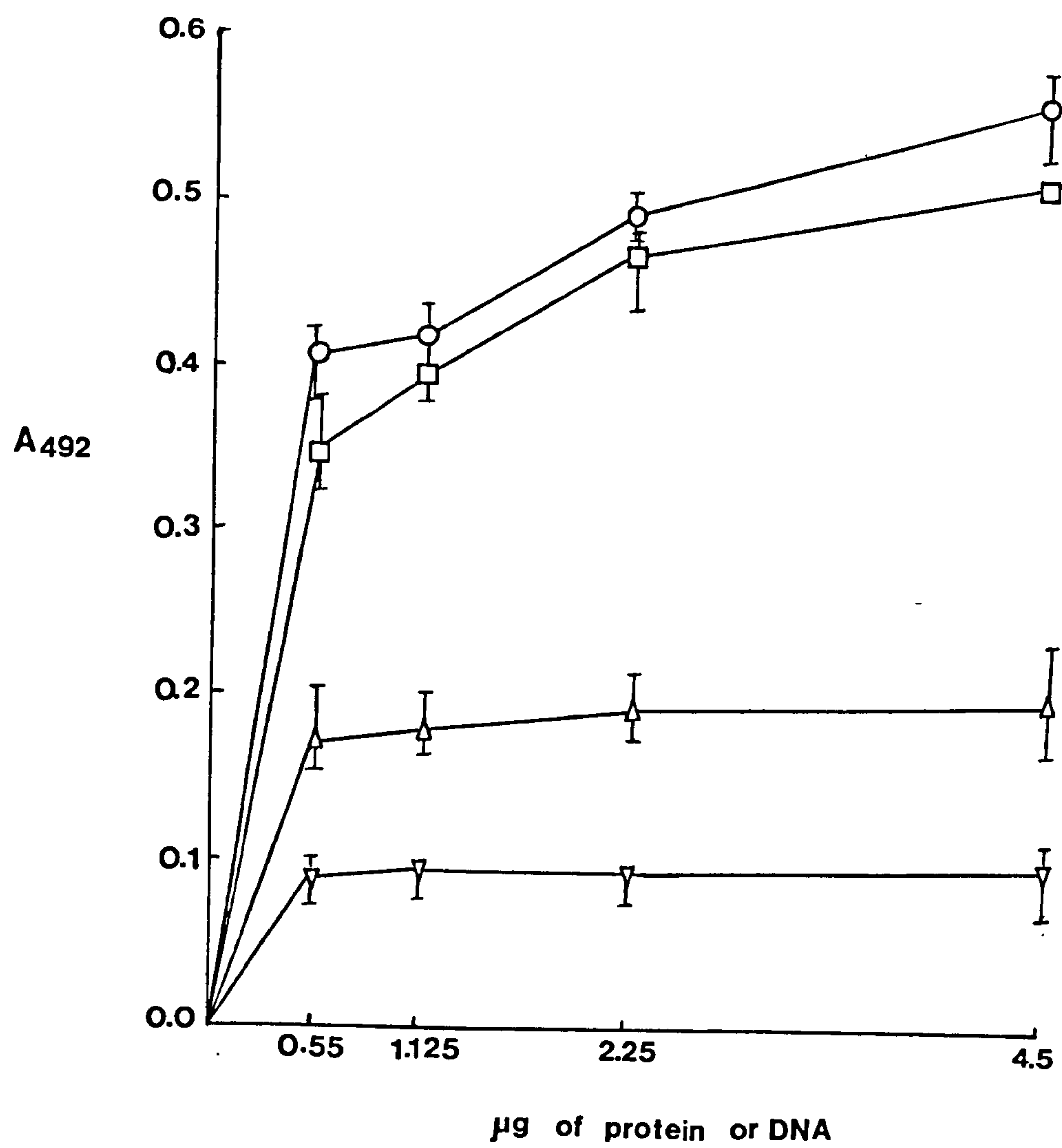




Figure 4.1.3

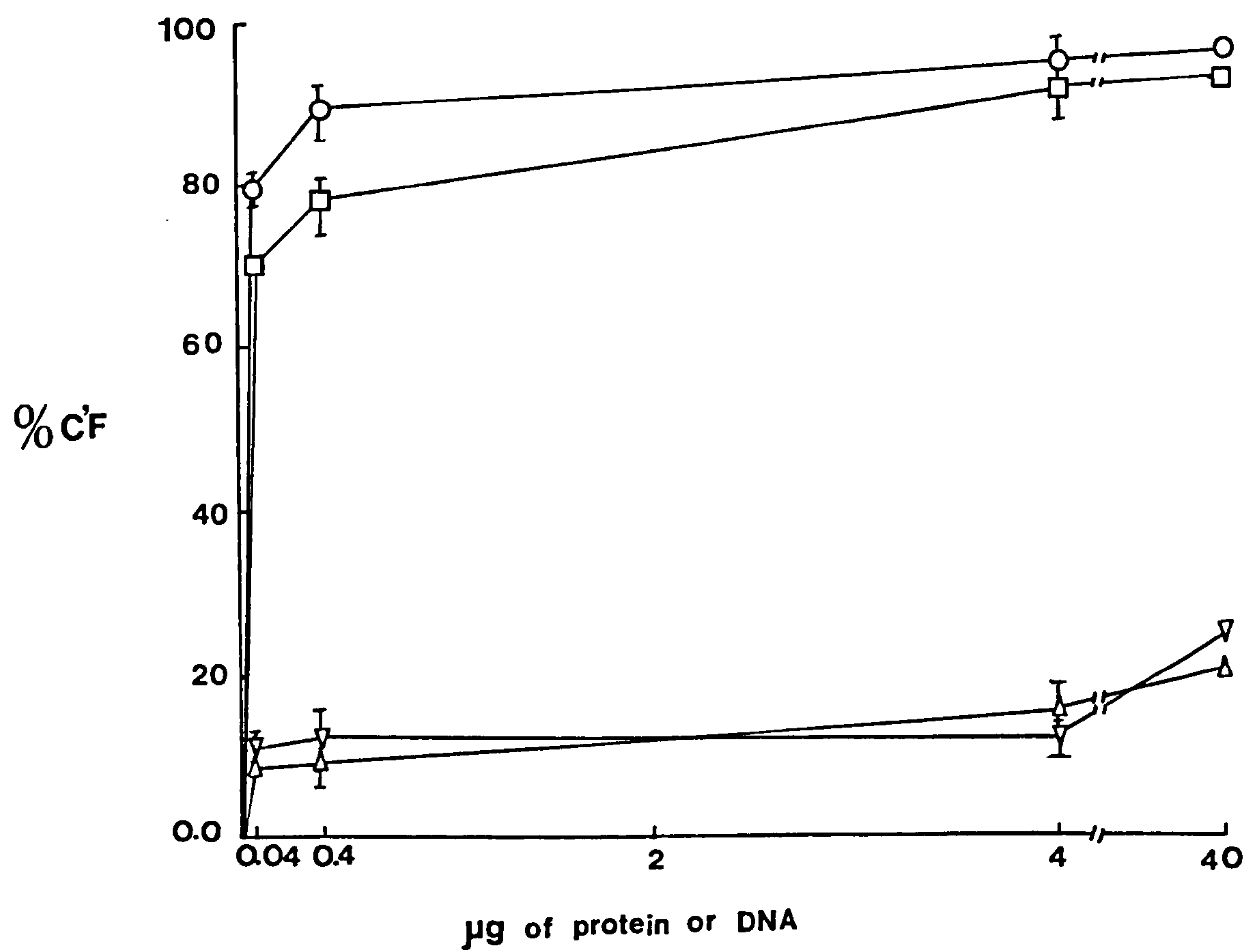
C'F Assay of R3.1 Antiserum with HeLa Digested- and Undigested-chromatins

Complement fixation assay of R3.1 antiserum (1/200) fixed with various concentrations of HeLa DNA, chromatin and micrococcal nuclease-digested chromatin before and after reconstitution with HeLa DNA. All chromatin preparations were measured as total protein content except DNA alone estimated as total DNA. (Antibody and antigen controls were subtracted).

- HeLa chromatin
- Micrococcal nuclease-digested HeLa chromatin reconstituted with HeLa DNA
- Δ Micrococcal nuclease-digested HeLa chromatin
- ∇ HeLa DNA alone

Antigen controls showed less than 3% C'F and antibody control exhibited only 1% C'F.

**Figure 4.1.3**



target antigen lies with the DNA itself or not, HeLa DNA alone was allowed to react with R3.1 antiserum. Figures 4.1.2 and 4.1.3 show a small amount of immunoreaction between R3.1 antiserum and HeLa DNA, when assayed by complement fixation and ELISA. This indicates that the target antigen is not the DNA alone but a complex of DNA and chromosomal proteins.

These data were substantiated by using the competition assay described in Section 3.4.5. R3.1 antiserum was absorbed either by HeLa chromatin or by micrococcal nuclease-digested chromatin prior to its application to an ELISA plate coated with HeLa chromatin. The results shown in Figure 4.1.4 demonstrate that a great deal of immunoreactivity of R3.1 antiserum (diluted 1/250) was absorbed by micrococcal nuclease-undigested HeLa chromatin even at a small amount of 0.625 $\mu$ g of chromatin in 50 $\mu$ l of the reaction mixture. The nuclease-digested chromatin failed to absorb large amounts of R3.1 antiserum. These data are presented clearly when plotted as a percentage of the absorbed chromatin in relation to the freely reacted R3.1 antiserum shown in Figure 4.1.4b.

In order to further characterise the protein antigens, HeLa chromatin and dehistonised chromatin together with cytoplasmic fractions were resolved on an SDS-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose paper as described in Section 3.6.4.1. The antibody-antigen reaction was monitored by enzyme linked immunoblotting as outlined in Section 3.5.4.1 and shown in Figure 4.1.5 (the bands are very faint on the photograph so a diagrammatic representation is traced from the original blot). R3.1 antiserum (1/200) did not show any reaction with cytoplasmic fraction (lanes 7 & 10) while faint colouring was noted corresponding to bands of Mr 45,000 and 43,000 on both dehistonised and chromatin fractions (lanes 5, 8, 6 & 9). Another faint band could be seen on the immunoblot in



Figure 4.1.4a      Competition ELISA of HeLa Chromatins Reaction with R3.1  
Antiserum

Enzyme linked immunoassay of R3.1 (1/250) absorbed with various concentrations of micrococcal nuclease-digested (as described in Section 3.8.2) and undigested HeLa chromatins, 50 $\mu$ l of this mixture then allowed to react with HeLa chromatin (layered as 10 $\mu$ g/well) immobilised on an ELISA plate. (Antigen and antibody controls were subtracted).

- Micrococcal nuclease-undigested HeLa chromatin
- Δ Micrococcal nuclease-digested HeLa chromatin

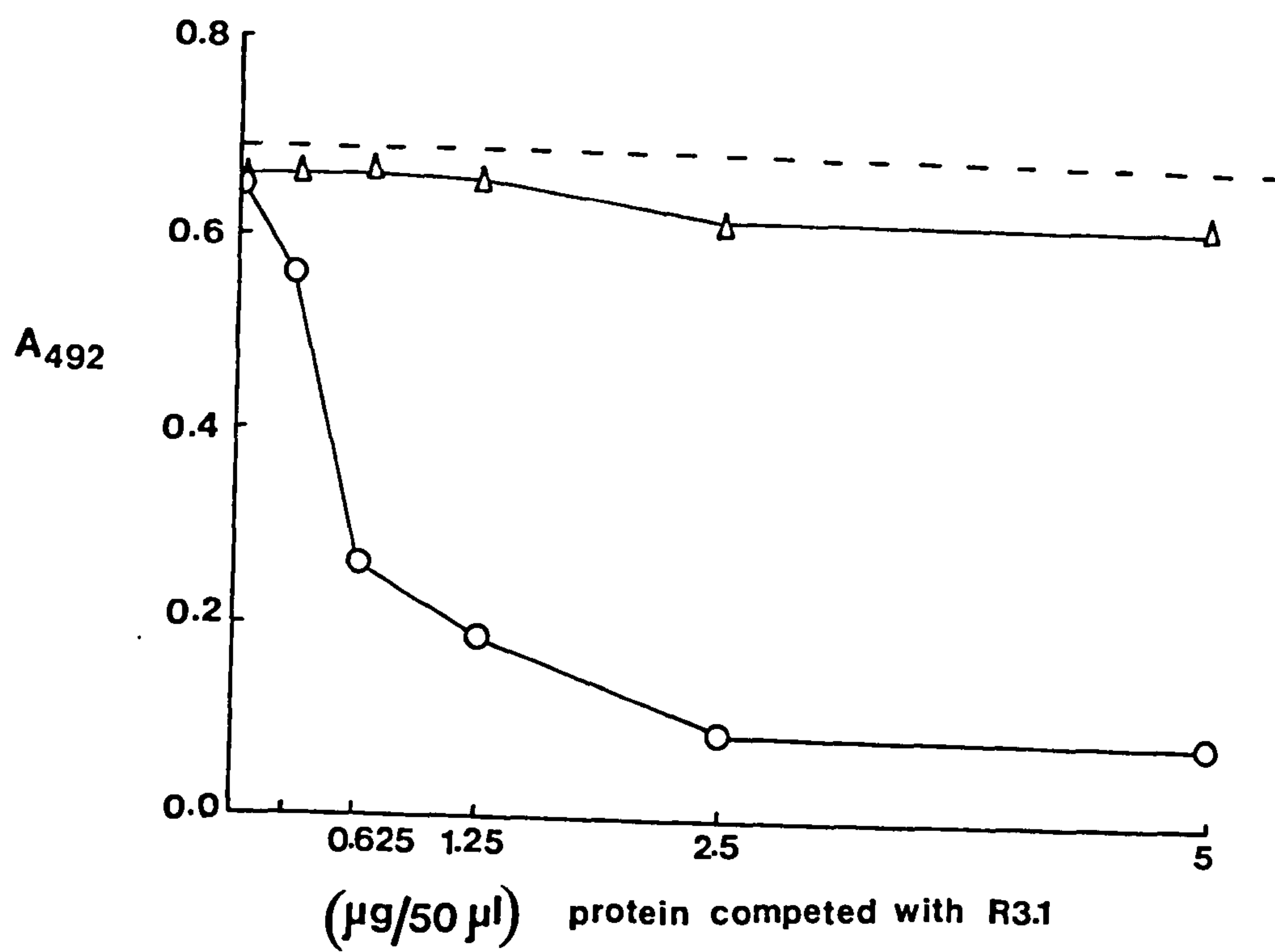
The dotted line represents the average of 50 $\mu$ l of unabsorbed R3.1 (1/250) reacted with HeLa chromatin (layered as 10 $\mu$ g/well) immobilised on ELISA plate.

Figure 4.1.4b      Percentage of Competition ELISA of HeLa Chromatins  
Reaction with R3.1 Antiserum

The amount of R3.1 (1/250) antiserum absorbed by micrococcal nuclease-digested and -undigested HeLa chromatin expressed as a percentage of free R3.1 reaction with immobilised HeLa chromatin (10 $\mu$ g/well).

- Micrococcal nuclease-undigested chromatin
- Δ Micrococcal nuclease-digested chromatin

**Figure 4.1.4a**



**Figure 4.1.4b**

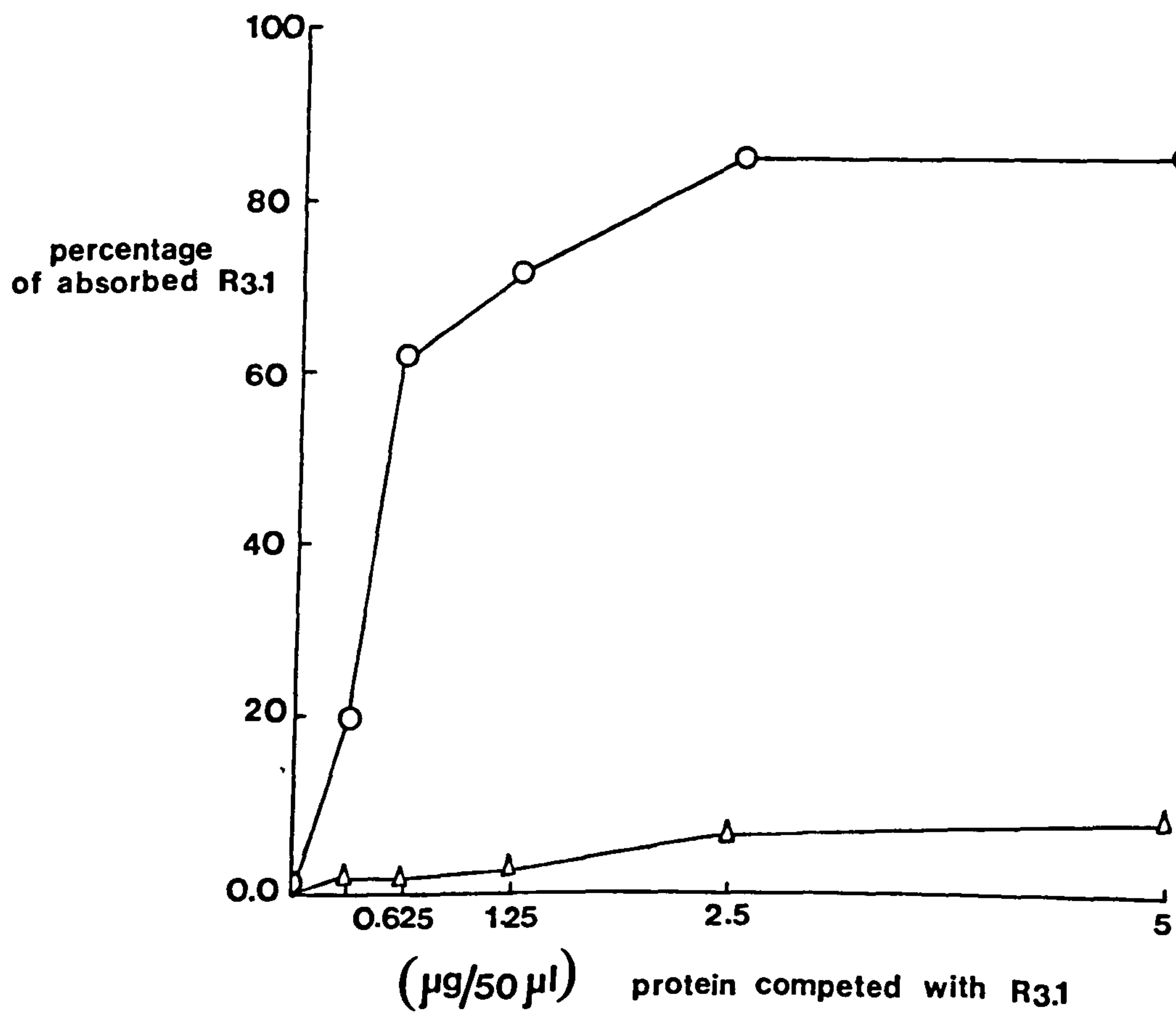




Figure 4.1.5

Immunoblots of HeLa Subcellular Fractions Reacted with  
R3.1 and Normal Rabbit Antisera

Protein immunoblot of R3.1 (1/200) antiserum (Strips B & C) and normal rabbit serum (1/200) (Strip D) reacted with cytoplasmic fraction (lanes 7,10 & 13), chromatin (lanes 6,9, & 12) and 2M-salt extracted chromatin (lanes 5,8, & 11) preparations from HeLa cells visualised by using the immunoperoxidase method described in Section 3.5.4.1. Strip (C) was incubated with HeLa DNA as described in Section 3.6.3.5 prior to its reaction with R3.1 antiserum. Lanes 2,3, & 4 represent the amido black-stained proteins corresponding to 2M-salt extracted chromatin, chromatin and cytoplasmic preparations. Lane (1) is the molecular weight marker. Phosphorylase b 94,000; Bovine serum albumin 67,000; Ovalbumin 43,000; Carbonic anhydrase 30,000; Soybean trypsin inhibitor 20,100;  $\alpha$ -Lactalbumin 14,400. The proteins were run in a 10% Laemmli gel containing 0.5M urea.



A

B

C

D

1

2

3

4

5

6

7

8

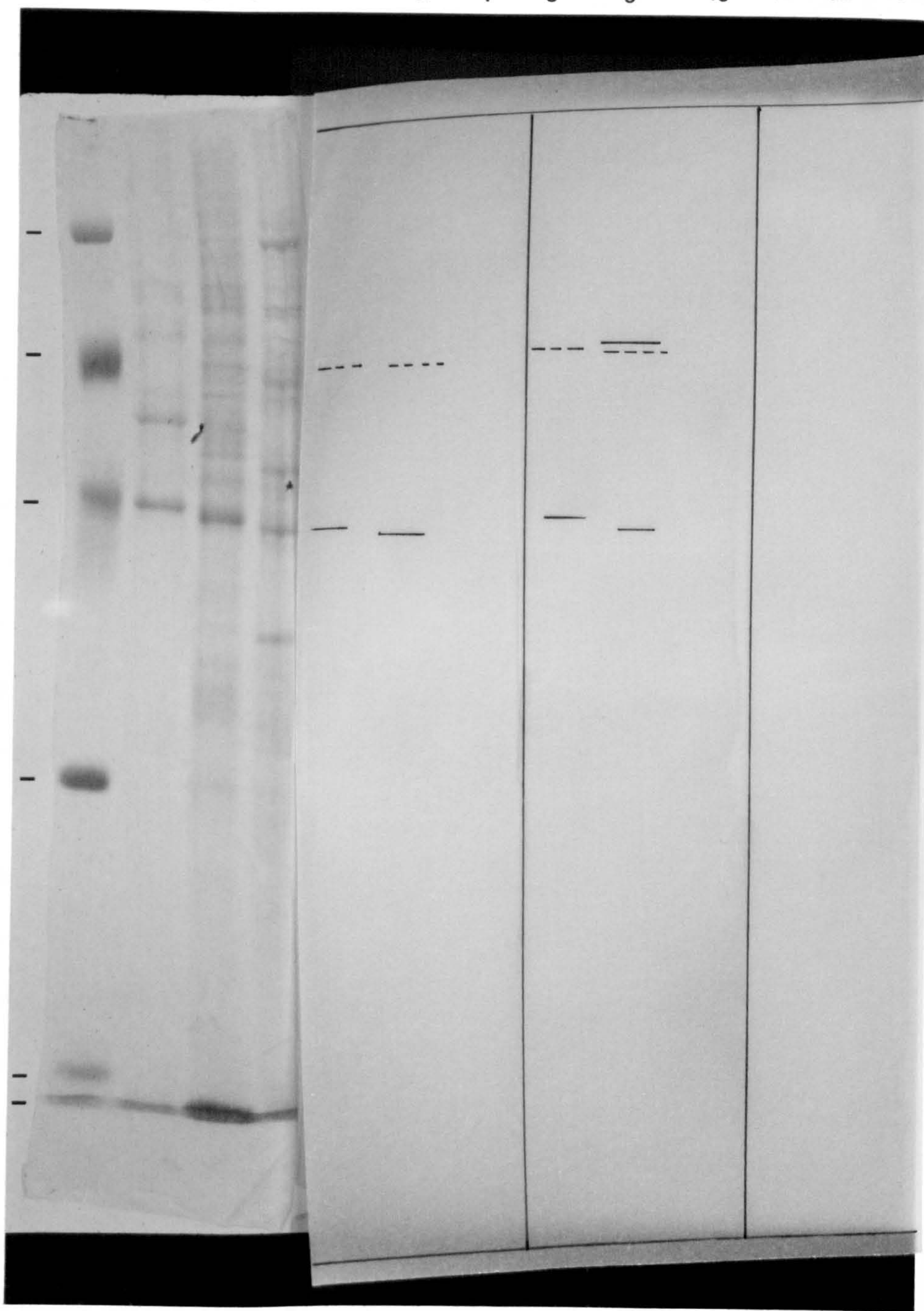
9

10

11

12

13



lanes 5 and 6 which represent the dehistonised chromatin and chromatin. This band is at an approximate molecular weight of 67,000. Increasing the concentration of the antiserum (R3.1) used, failed to show any increase in colour intensity on the immunoblot. Furthermore, normal rabbit serum (1/200) from unimmunised animal showed no visible immunoreaction on the blot (Strip D).

Since the antigens in HeLa chromatin require the presence of DNA for its reactivity with R3.1 antiserum, and the DNA itself is too large a molecule to enter the high percentage polyacrylamide gel, incubating the blot with DNA may enhance or change the antigenic determinants on the blot and make them accessible to the antibody recognition. The blot was incubated with HeLa DNA (100µg/ml) prior to its reaction with R3.1 antiserum as described in Section 3.6.3.5. The pattern seen after DNA treatment did not significantly affect the distribution of the reactive proteins except for the appearance of one faint band (Mr 70,000) in the dehistonised fraction and two faint bands at Mr 70,000 and 68,000 in the chromatin lane (Lane 9). The latter may be the same as the 67,000 seen in lanes 5 and 6.

By inspecting the immunoblot in Figure 4.1.5, it is possible to categorise the proteins which reacted with R3.1 antiserum into two groups. The first comprises the 67,000-70,000 proteins seen in chromatin and the dehistonised fractions in the DNA-treated blot. The second group comprises the 43,000-45,000 proteins detected in the chromatin and dehistonised chromatin, respectively, of both the DNA-treated and untreated blots. This group is therefore immunologically detectable in all chromatin and dehistonised chromatin except those which were allowed to react with normal rabbit serum.

It is interesting to see the appearance of 68,000 and 70,000 protein bands in the DNA-treated blot (Lanes 8 & 9) which indicates the necessity of DNA for the antibody-antigen reaction of these proteins.



It is worth mentioning that the immunoreactivity of HeLa chromatin with R3.1 was greatly affected by SDS-treatment. It was observed that this reactivity reduced drastically when chromatin preparation was incubated with 1% SDS in TN buffer for 90 minutes at room temperature then applied onto a nitrocellulose paper and allowed to react with R3.1 in the same way as for the electroblot. The reactivity decreased further upon increasing the SDS concentration until it was completely abolished at 10% SDS treatment. This was monitored visually by looking at the intensity of the substrate colour on the reacted chromatin.

To determine the localisation and distribution of the antigen using R3.1 antiserum as first antibody, an indirect fluorescein isothiocyanate (FITC) staining of HeLa cells was performed (Figure 4.1.6). The staining is seen mainly in the nuclei with more intensity at the periphery (perinuclear area). A considerable amount of staining could also be noted in the cytoplasm.

Pure nuclei were also prepared as described in Section 3.1.1.1 and then fixed on the slide and treated with R3.1 antiserum followed by FITC-conjugated second antibody in the same manner as the treatment of the cells. Again all the nuclei were stained with more intensity at the nuclear periphery (Figure 4.1.7). In comparable experiments using the same technique, Chang liver and FL cells failed to show significant nuclear reaction with R3.1. A small amount of cytoplasmic reaction was again observed (Figure 4.1.8). Moreover, normal rabbit serum used at the same dilution as R3.1 antiserum showed no significant reaction with HeLa cells (Figure 4.1.9).

As is observed in Figure 4.1.6a, fluorescein stained cells showed more staining intensity on the periphery of the nuclei. This suggests that part of the reaction antigen may be associated with nuclear matrix or nuclear envelope. This observation, together with the fact



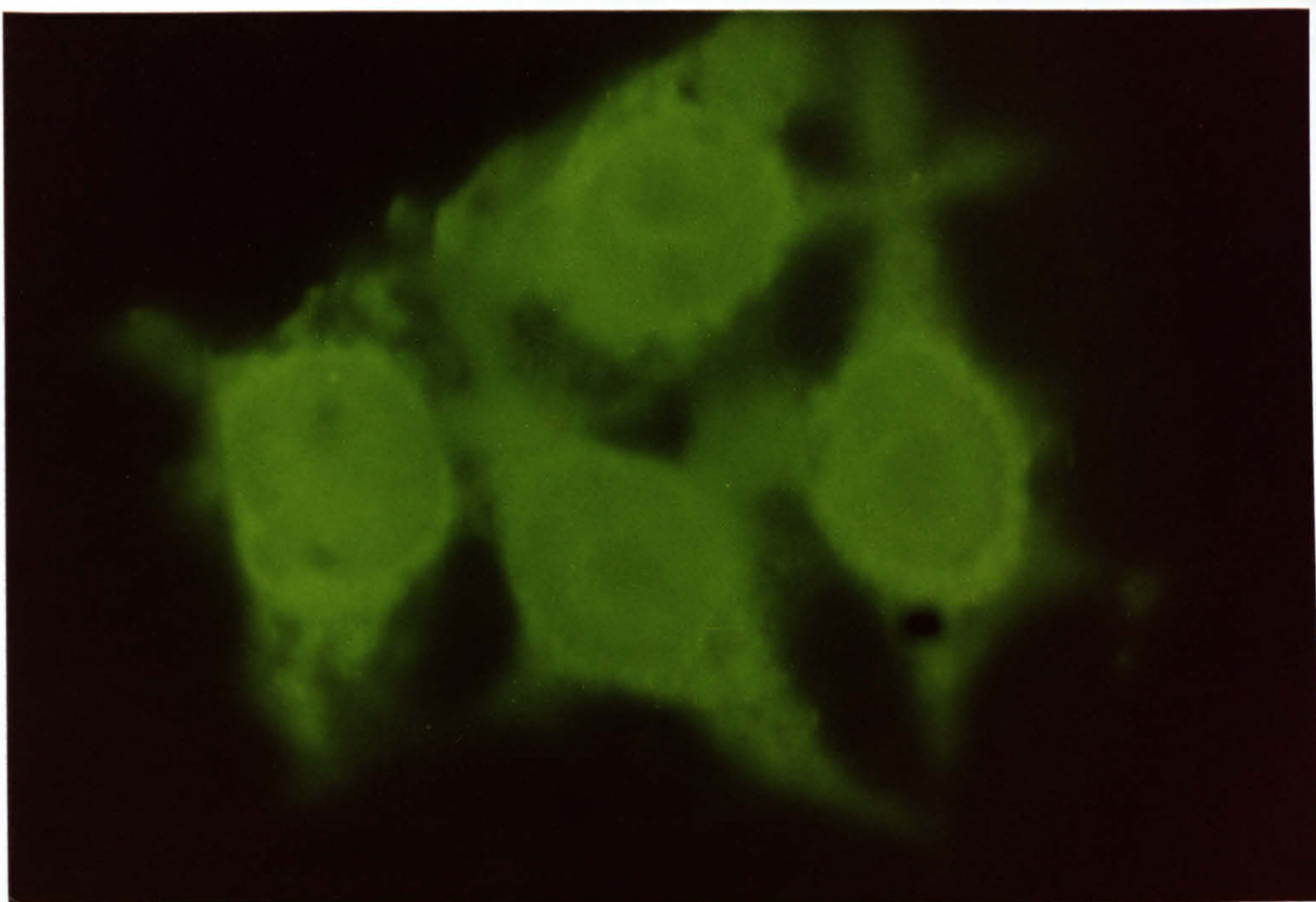
Figure 4.1.6

FITC-staining of HeLa Cells Reacted with R3.1 Antiserum

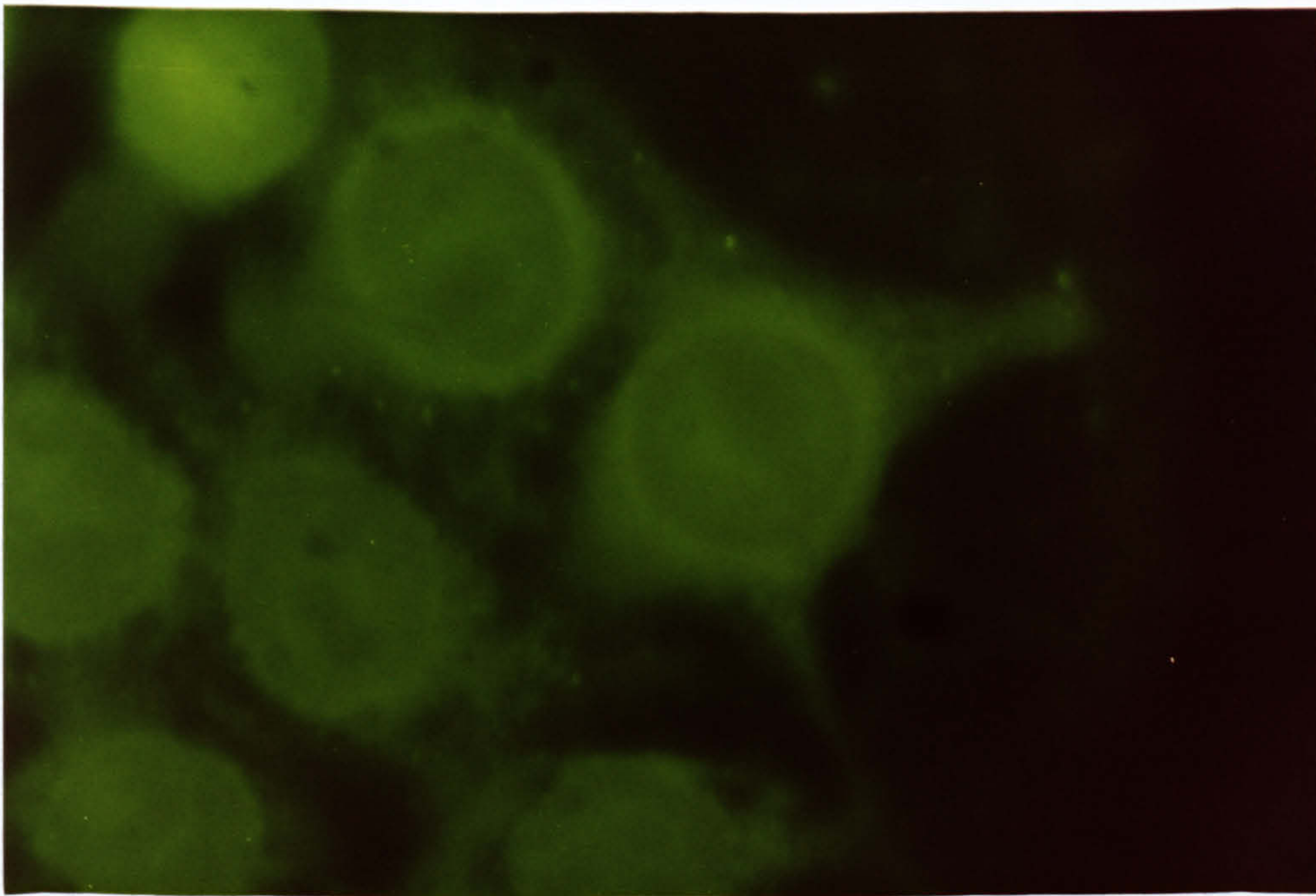
(a,b,c) Immunofluorescent staining of HeLa cells  
reacted with 1/100 R3.1 antiserum (X2000).



a



b



c

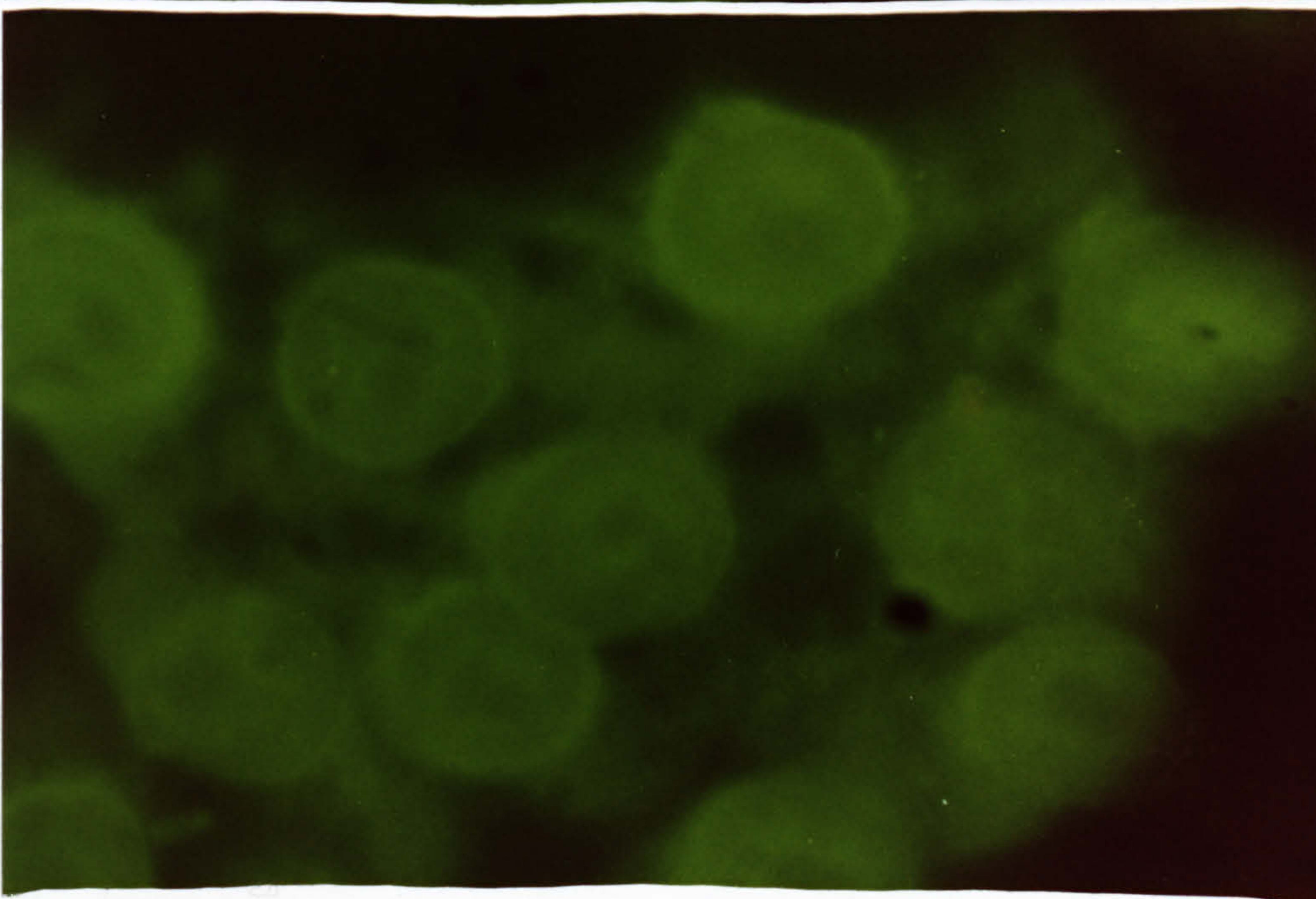




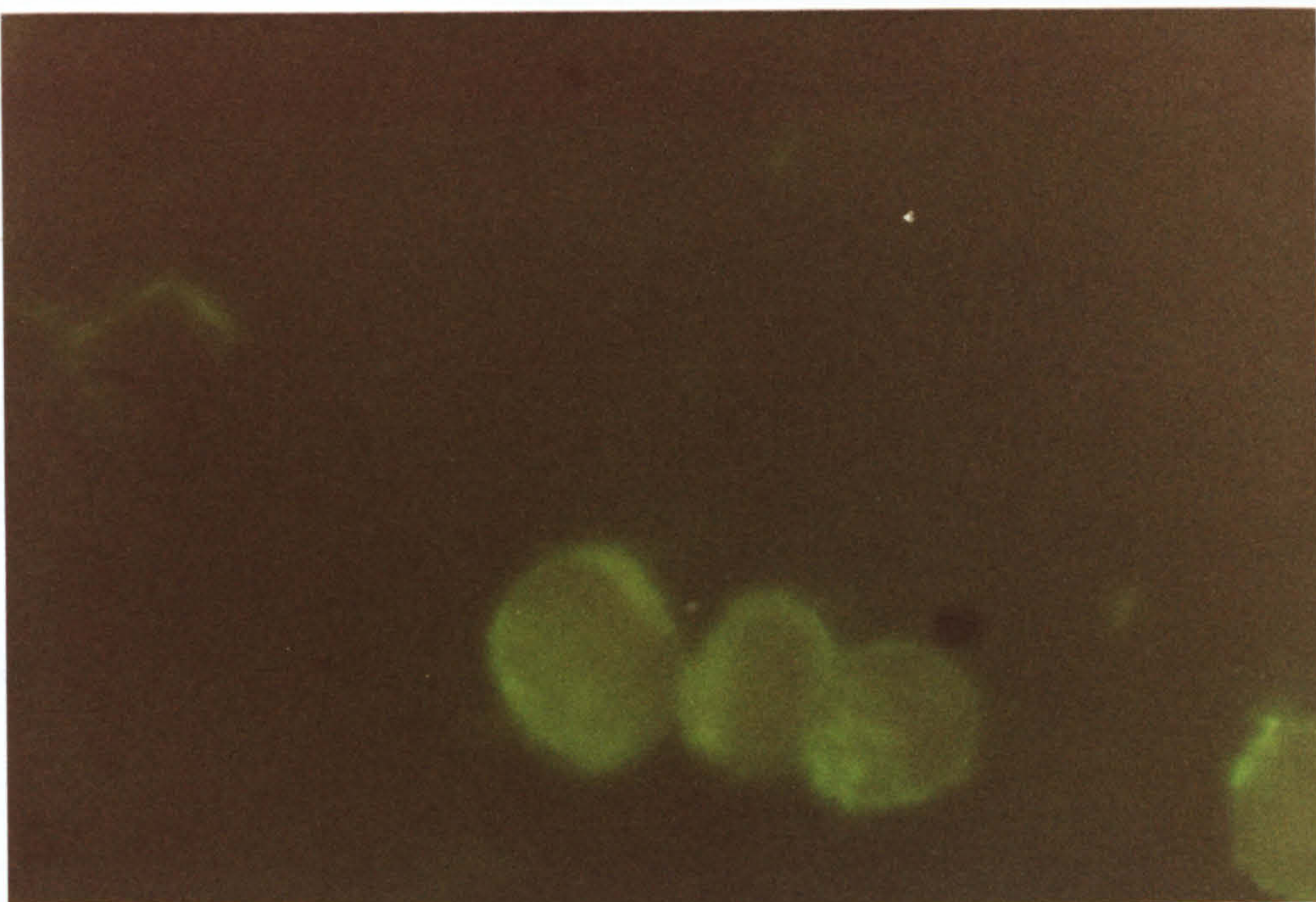
Figure 4.1.7

FITC-staining of HeLa Nuclei Reacted with R3.1 Antiserum

- (a) Indirect immunofluorescent staining of HeLa nuclei reacted with R3.1 antiserum (X2000).
- (b) Phase contrast of the nuclei in (a).



**a**



**b**

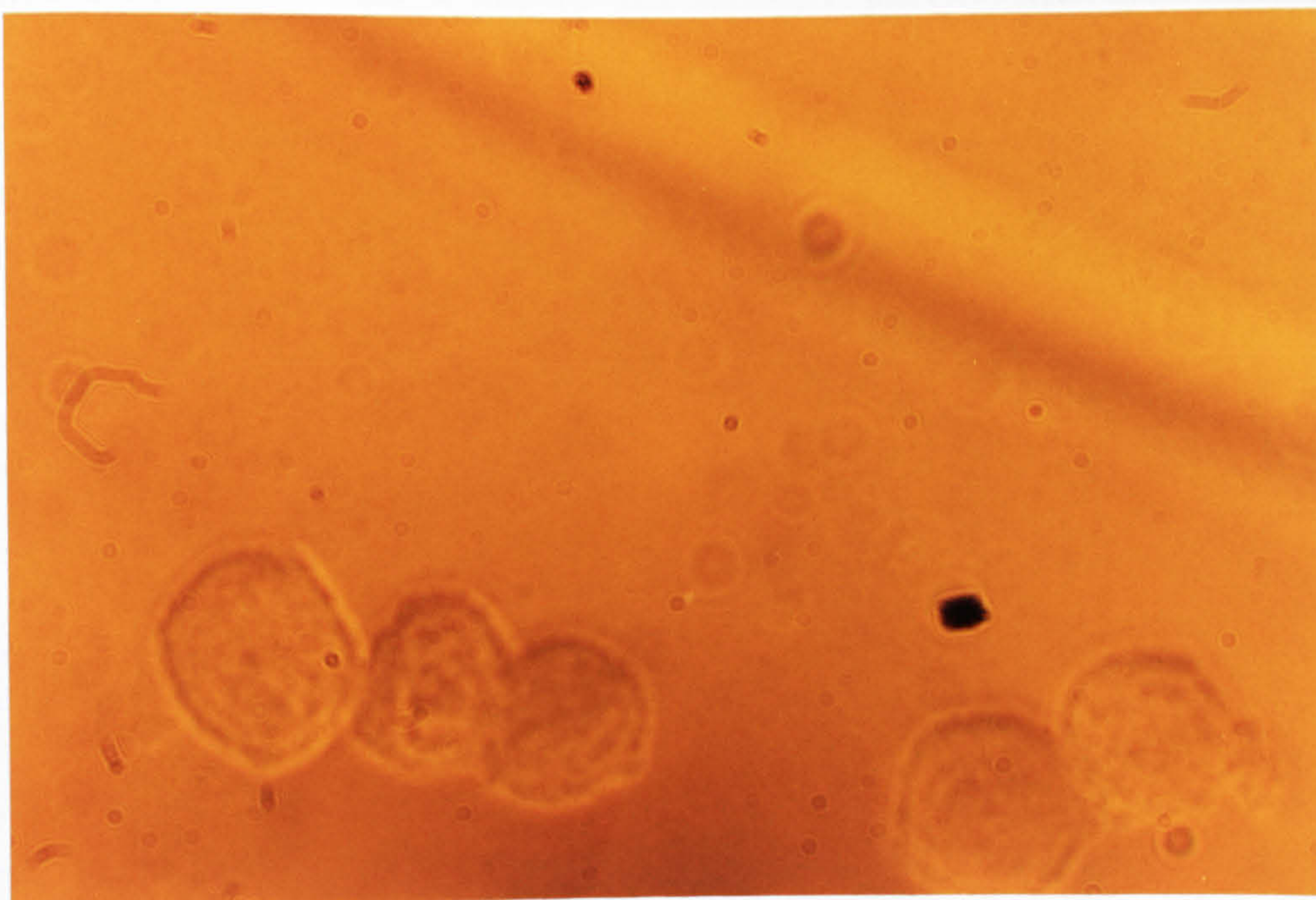




Figure 4.1.8

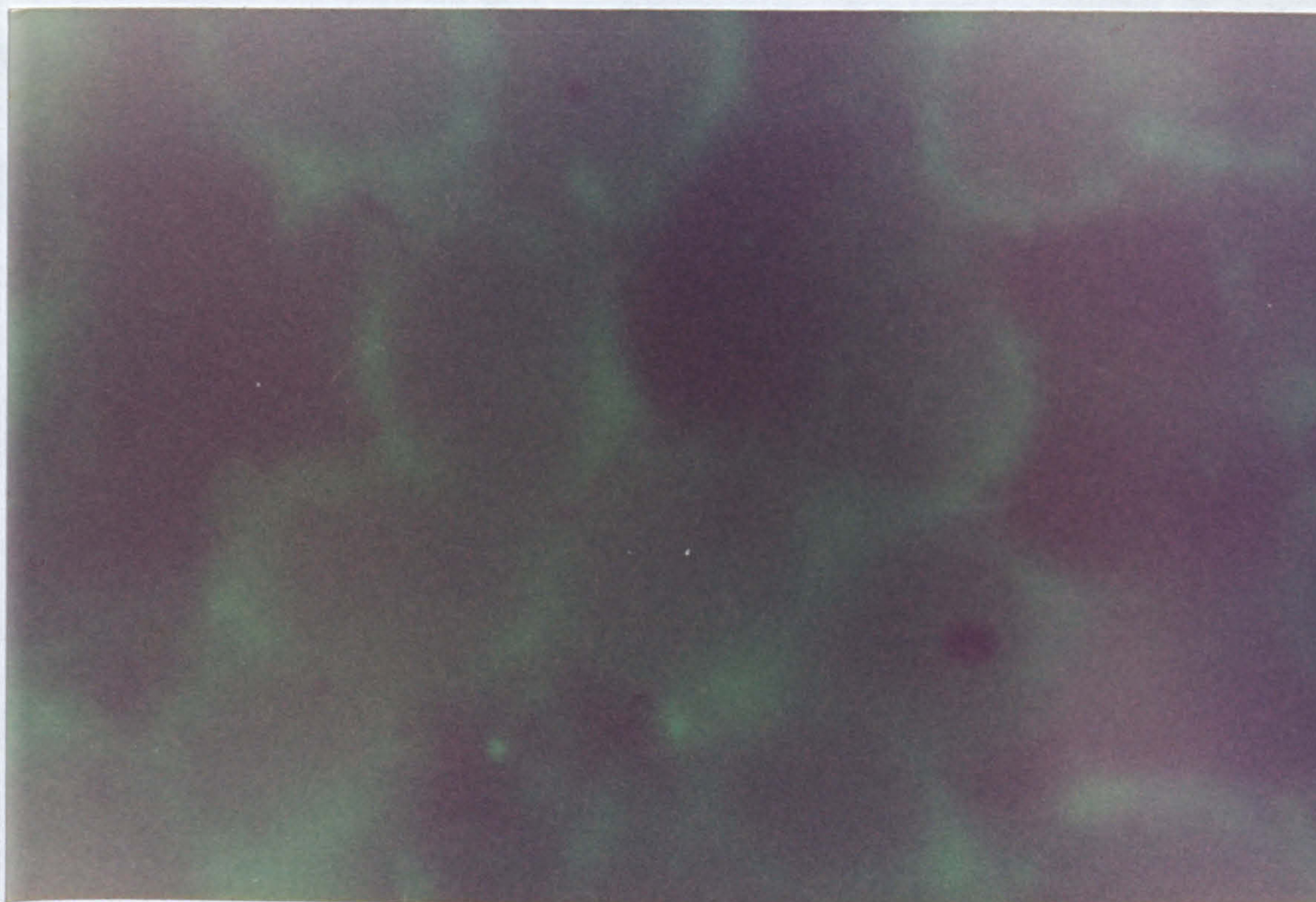
FITC-staining of Human Cells Reacted with R3.1 Antiserum

Indirect immunofluorescent staining of human cells  
allowed to react with R3.1 antiserum (X2300).

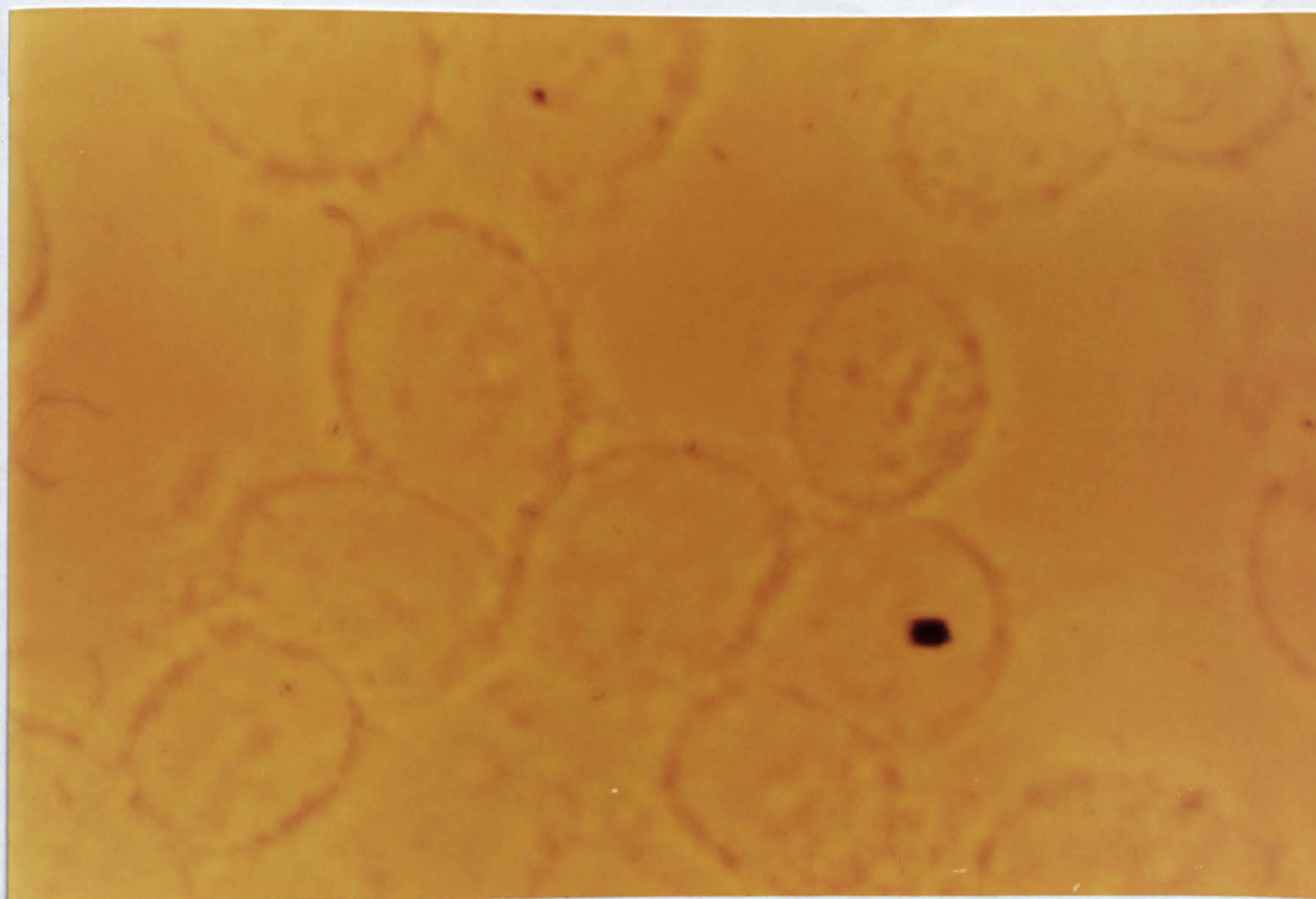
- (a) Chang liver cells
- (b) Phase contrast of (a)
- (c) FL cells
- (d) Phase contrast of (c)



**a**

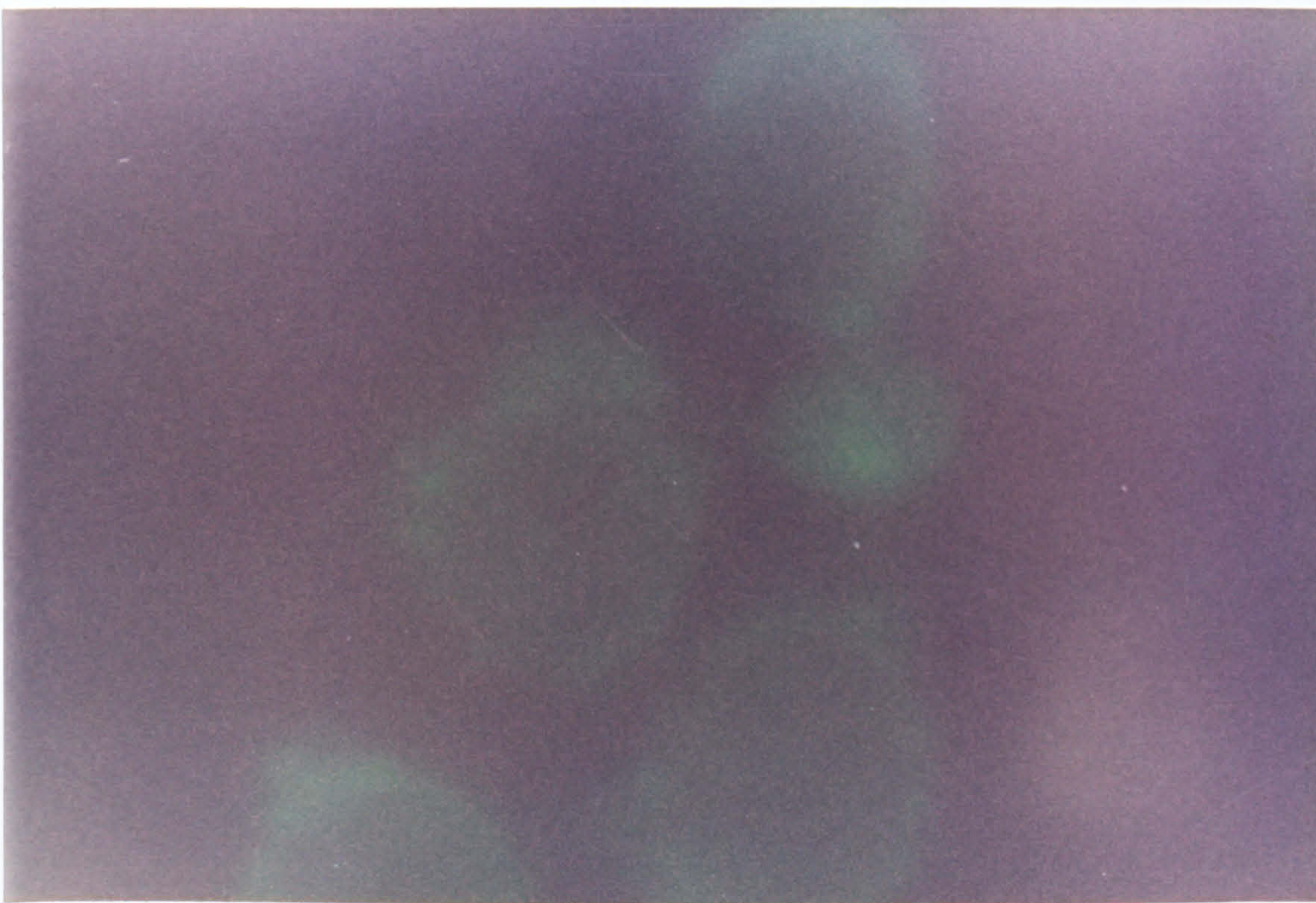


**b**





c



d

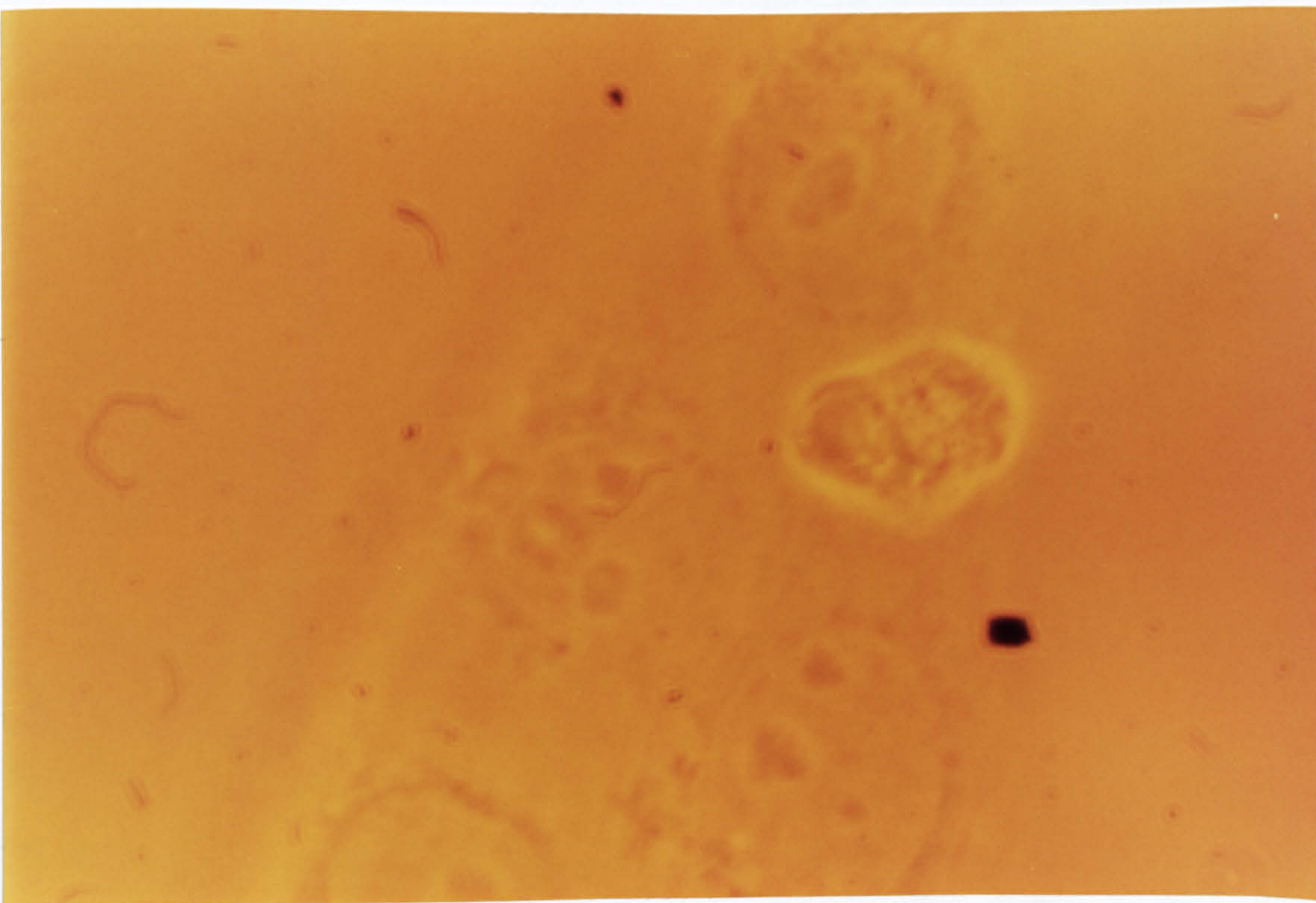




Figure 4.1.9

FITC-staining of HeLa Cells Reacted with Normal Rabbit  
Serum

Indirect immunofluorescent staining of normal rabbit  
serum (1/100) allowed to react with HeLa cells (X2000).



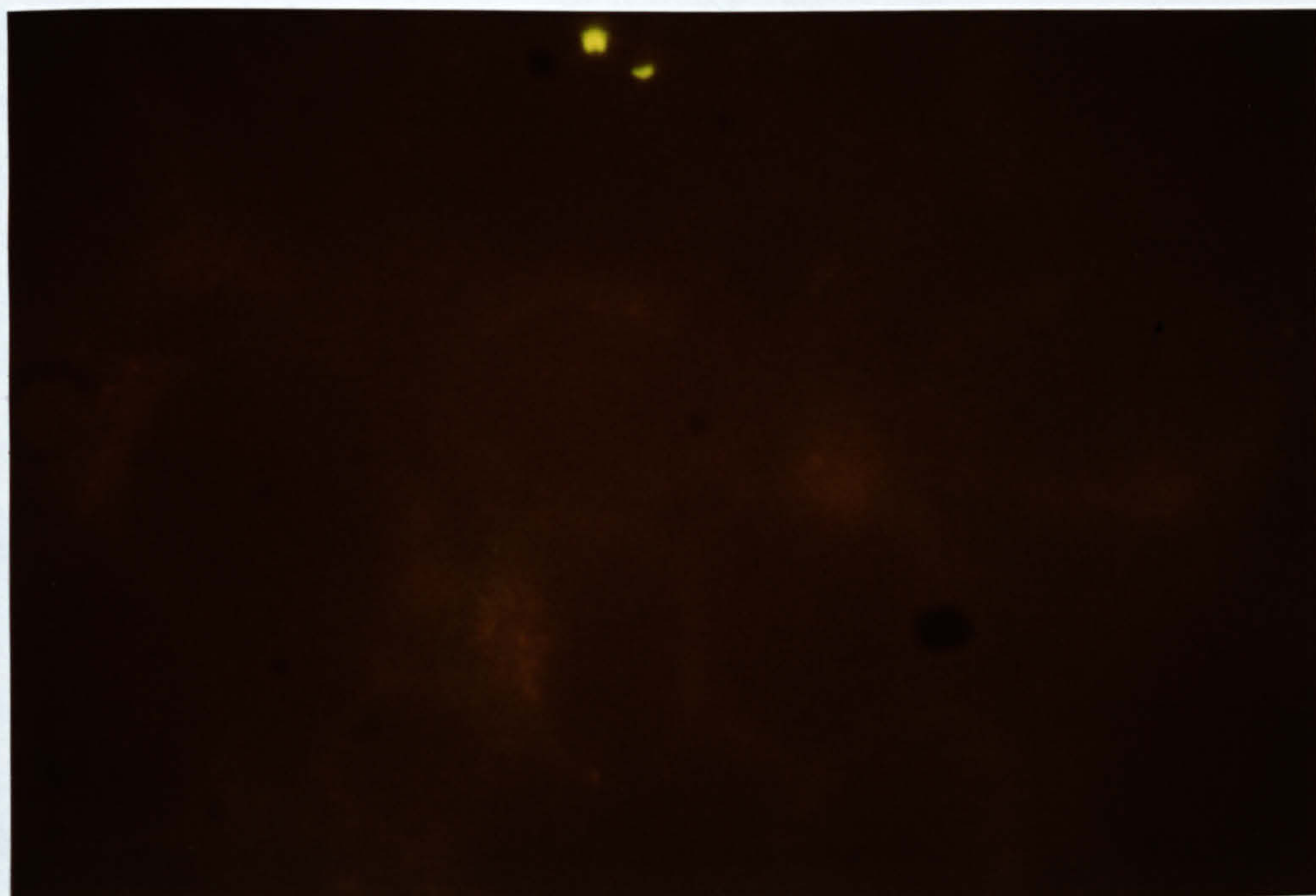
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... bands of Mr 68,000-70,000 showed some degree of reactivity  
... antiserum in the presence of DNA suggests that nuclear matrix  
... complex-lamina proteins may be the dominant antigens since these  
... molecular weight range of 68,000-70,000. Therefore, the  
... fraction from a nuclear pore complex-lamina-enriched  
... (prepared as described in Section 2.2.2) was allowed to react  
... antiserum (1/250). ... of ...  
... nuclear lamina preparation ...



... no significant ...  
... allowed to react with ...  
... the enzyme linked immunosorbent assay (Figure 4.1.12a & b), ...  
... showed a reaction with ... relatively higher than  
... with other human and non-human chromatin. Again, ...  
... cells showed rather high reactivity with ... This  
... is even higher than that detected by C-F assay. The  
... in the reactivity between ELISA and C-F assay will be  
... later.

It is worth noting that the human cells ...  
... fixation and ELISA assay ...



that protein bands of Mr 68,000-70,000 showed some degree of reactivity with R3.1 antiserum in the presence of DNA suggests that nuclear matrix or pore complex-lamina proteins may be the dominant antigens since these lie in the molecular weight range of 68,000-70,000. Therefore, the protein fraction from a nuclear pore complex-lamina matrix-enriched preparation (prepared as described in Section 3.8.7) was allowed to react with R3.1 antiserum (1/250). Equal amount of proteins from chromatin and nuclear lamina preparations layered on an ELISA plate showed different reactivity towards R3.1 antiserum (Figure 4.1.10). Nuclear lamina-matrix proteins exhibited only about half of the reactivity seen with a chromatin preparation tested simultaneously. It is worth noting that the lamina-matrix preparation involved the use of small amounts of micrococcal nuclease digestion.

The specificity of R3.1 antiserum was tested with human and non-human chromatins (Figure 4.1.11a & b) using C'F assay. The antiserum showed more than 95% complement fixation at a dilution of 1/200 when reacted with HeLa chromatin, while little (less than 10%) of the complement was fixed on its reaction with other human chromatins. Approximately 15-20% fixation was noted with Chang liver and FL cells. Similarly, no significant complement fixation was observed when R3.1 antiserum was allowed to react with non-human chromatins (Figure 4.1.11b).

In the enzyme linked immunoassay (Figure 4.1.12a & b), R3.1 antiserum showed a reaction with HeLa chromatin relatively higher than its reactivity with other human and non-human chromatins. Again, Chang liver and FL cells showed rather high reactivity with R3.1. This reactivity is even higher than that detected by C'F assay. The difference in the reactivity between ELISA and C'F assays will be discussed later.

It is worth noting that the human cell lines used for the microcomplement fixation and ELISA assays were from various tissues,

Figure 4.1.10

ELISA of R3.1 Antiserum with HeLa Lamina Preparation

An enzyme linked immunosorbent assay comparing the reactivity of HeLa chromatin and HeLa nuclear-lamina-matrix-enriched preparation towards R3.1 (1/250) antiserum. (Antibody and antigen controls were subtracted).

- HeLa chromatin
- HeLa nuclear pore complex-lamina-matrix preparation

Antibody control was found to be 0.04 A<sub>492</sub> unit while the highest antigen control was 0.14 A<sub>492</sub> unit.



Figure 4.1.10

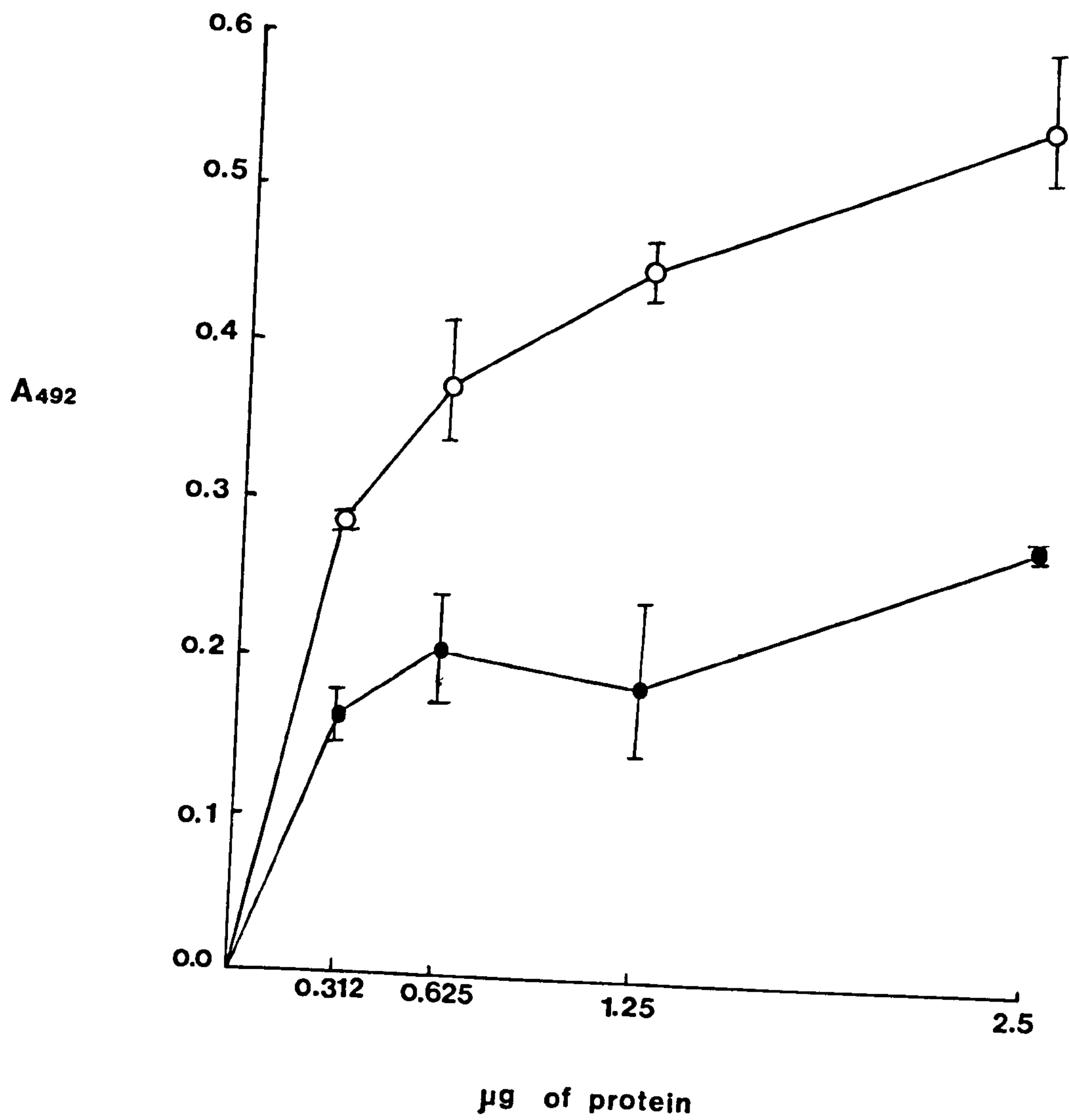


Figure 4.1.11a

C'F Assay of R3.1 Antiserum With Human Chromatin

Complement fixation assay of various human chromatin  
(20µg of protein) from different tissues (see Table 2a)  
fixed with different concentrations of R3.1  
antiserum. (Antibody and antigen controls were  
subtracted).

- HeLa
- Chang liver
- Δ K562
- ▽ KB
- ▲ FL
- HT1080

Antigen control showed between 2-3% C'F while antibody controls exhibited only  
1% C'F at 1/200 dilution and a -1% C'F at 1/1600 dilution of antibody.

**Figure 4.1.11a**

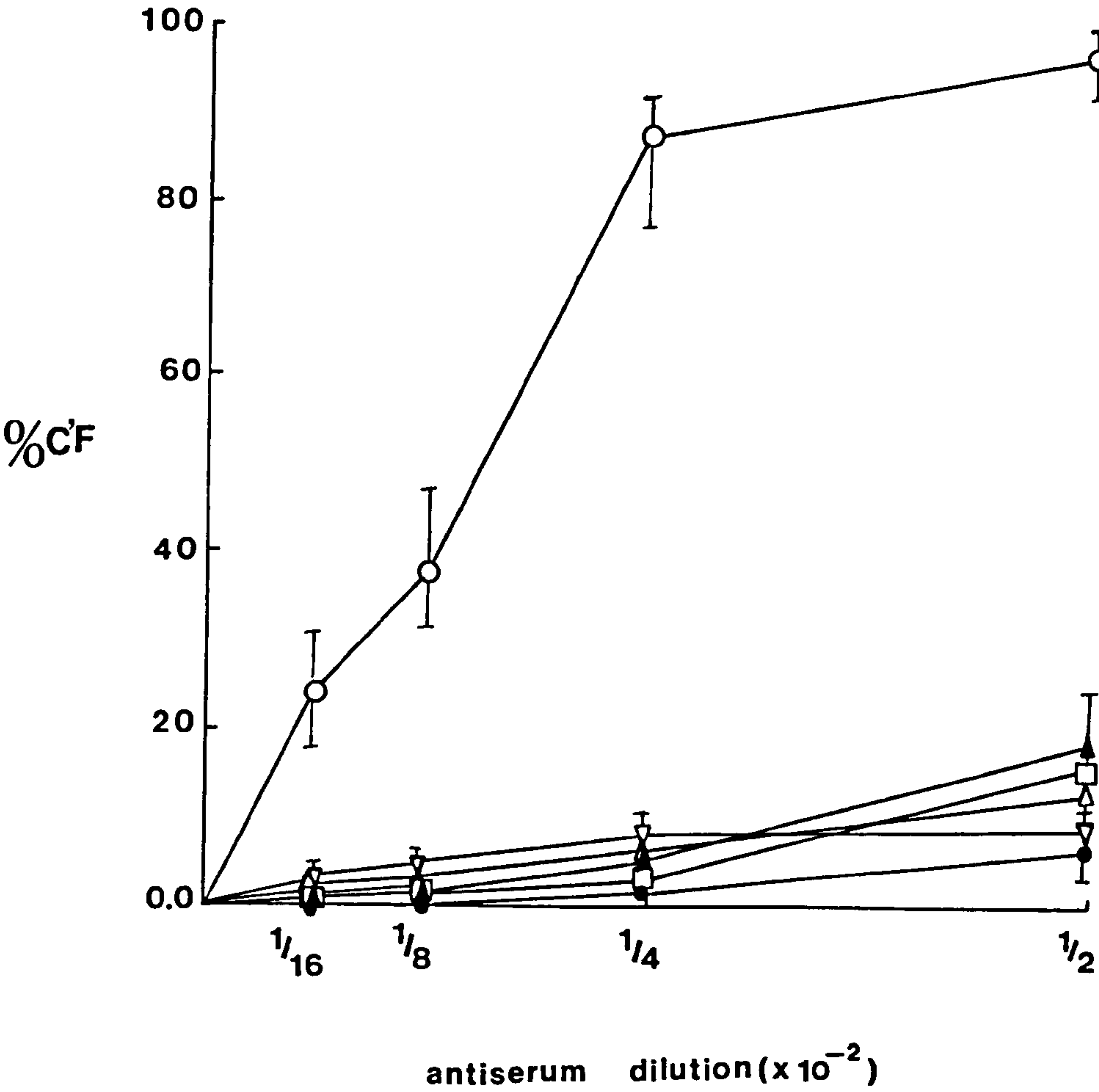




Figure 4.1.11b      C'F Assay of R3.1 Antiserum with Non-human Chromatins

Complement fixation assay of HeLa chromatin compared with various non-human chromatins (20 $\mu$ g of protein) from different tissues (see Table 2b) fixed with different concentrations of R3.1 antiserum. (Antibody and antigen controls were subtracted).

- HeLa
- △ Rat liver
- BSC-1
- ▲ L929

**Figure 4.1.11b**

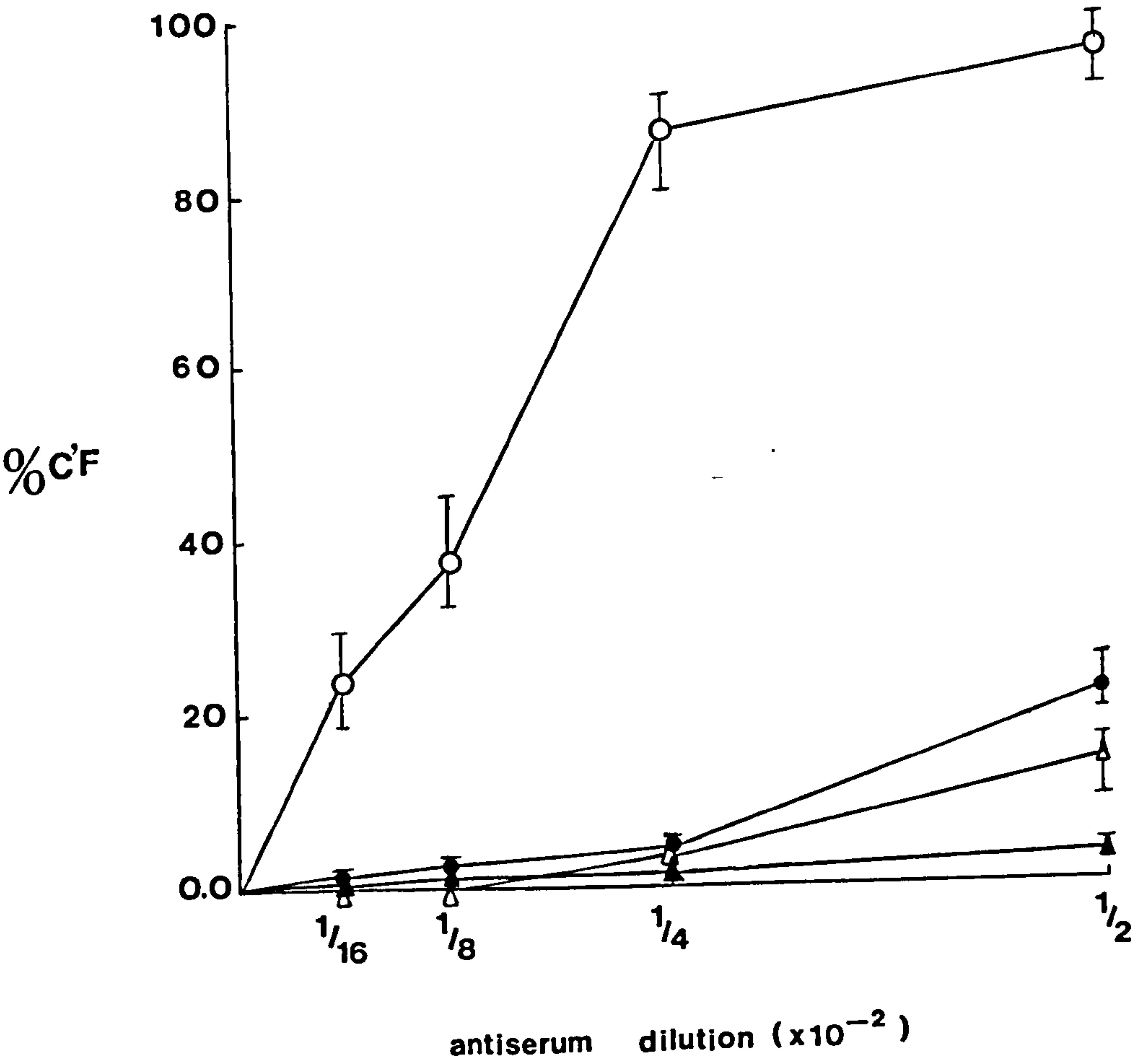


Figure 4.1.12a

ELISA of R3.1 Antiserum with Human Chromatins

An enzyme linked immunosorbent assay of different concentrations of R3.1 antiserum reacted with fixed amounts of human chromatins (10 $\mu$ g chromatin layered in each well) immobilised on an ELISA plate. (Antibody and antigen controls were subtracted).

- HeLa
- H.Ep-2
- Chang liver
- △ K562
- ▽ KB
- ▲ FL
- HT1080

Antigen controls were as follows: HeLa 0.149, H.Ep-2, 0.18, Chang liver 0.16, K562 0.145, KB 0.15, FL 0.15 and HT1080 0.16 A<sub>492</sub> unit. Antibody controls were less than 0.05 A<sub>492</sub> unit.



**Figure 4.1.12a**

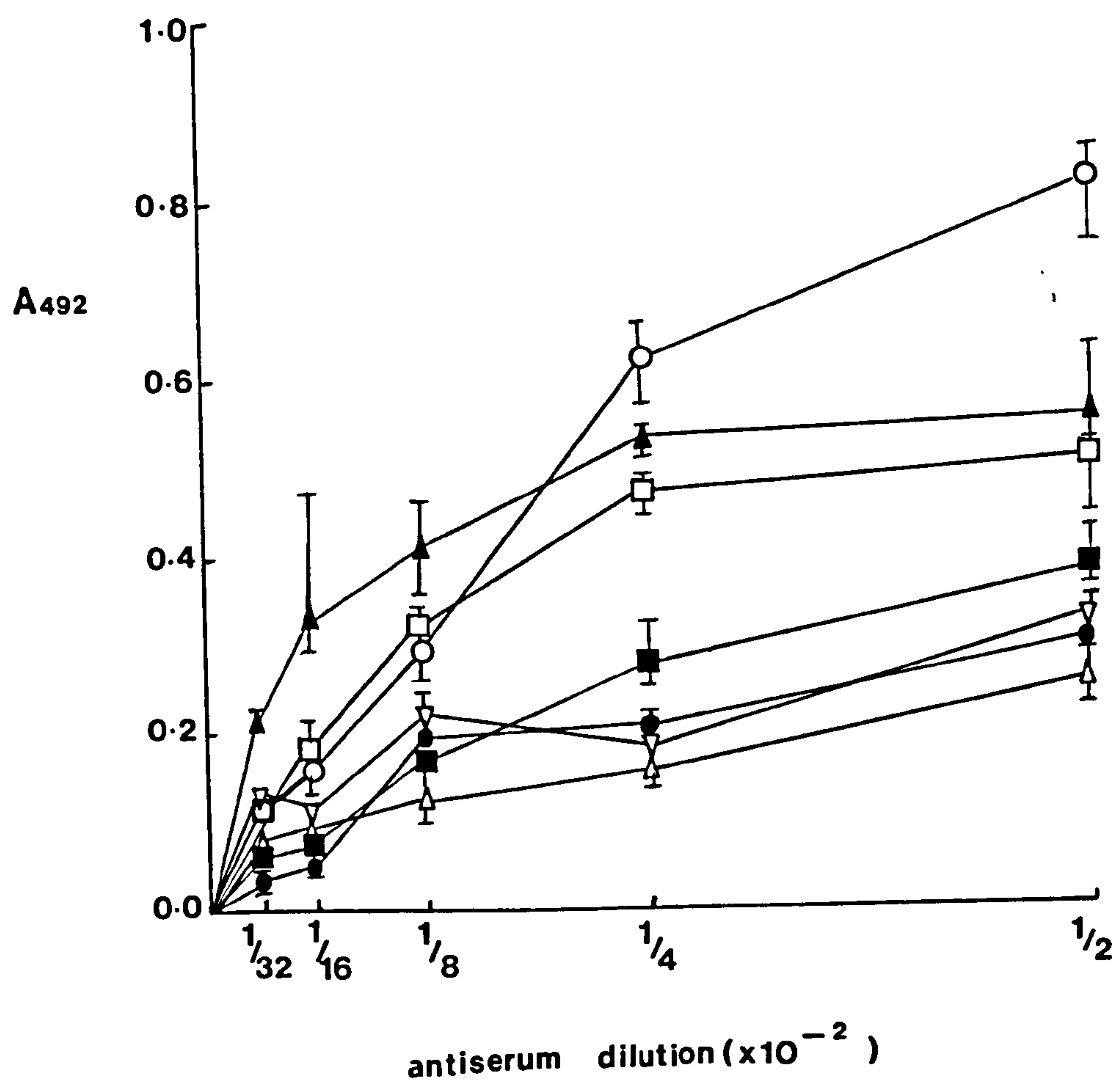


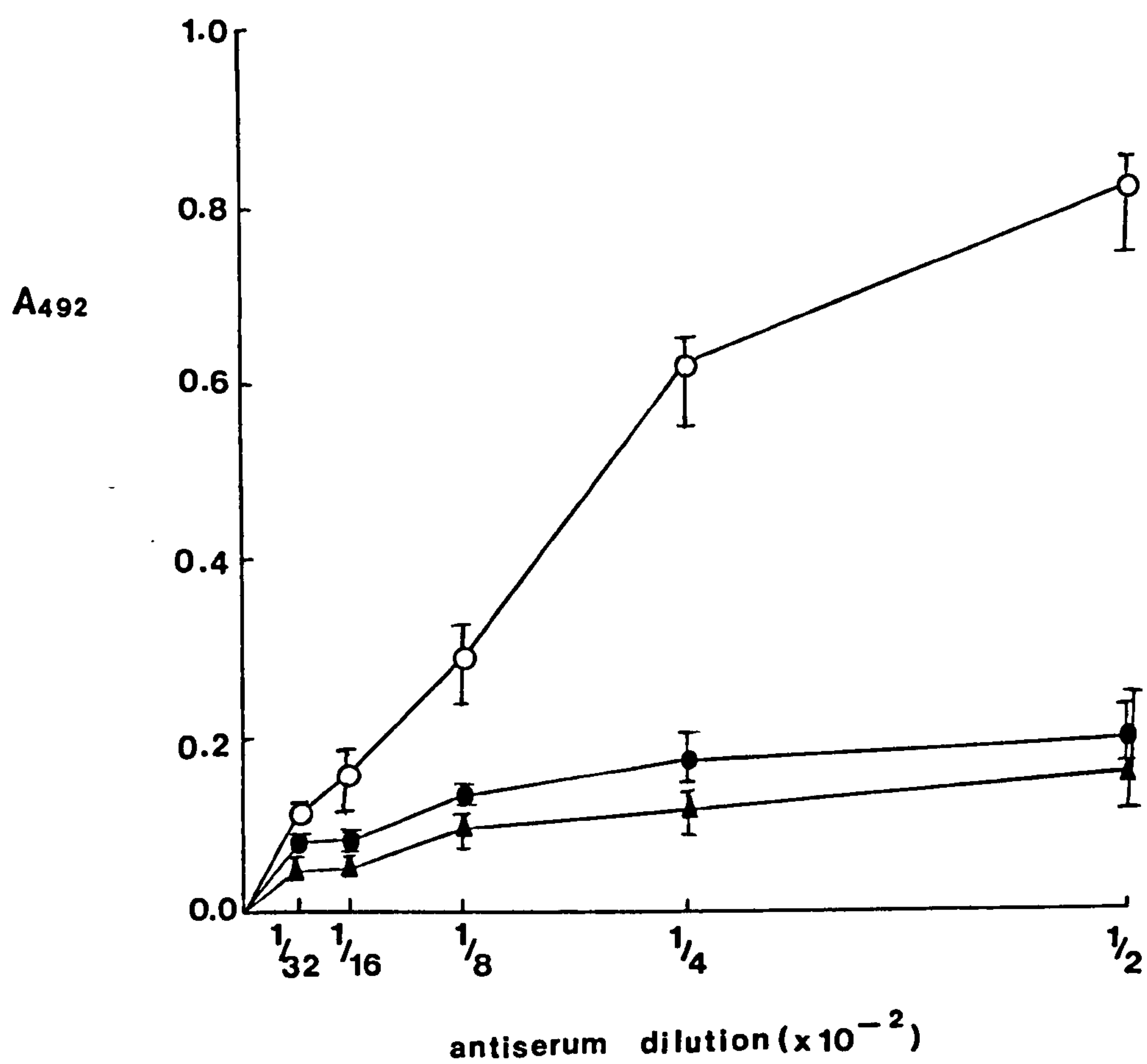
Figure 4.1.12b

ELISA of R3.1 Antiserum with Non-human Chromatins

An enzyme linked immunosorbent assay of different concentrations of R3.1 antiserum reacted with non-human chromatins (10 $\mu$ g layered in each well) immobilised on an ELISA plate. The reactivity simultaneously compared with equal amount of HeLa chromatin. (Antibody and antigen controls were subtracted).

- HeLa
- BSC-1
- ▲ L929

Figure 4.1.12b





while the non-human chromatins were not only from different tissues but also from three kinds of animals; rat, monkey and mouse (see Table 2a & b).

Since FL and Chang liver cells have shown relatively higher immunoreactivity with R3.1 compared with other human cell lines, and in addition, some kind of complex association between the DNA and HeLa chromatin has been found necessary for the immunoreactivity with R3.1 antiserum, it is obviously important to compare the DNA content of each chromatin preparation with the degree of its reactivity with R3.1 antiserum. Table (5) shows the ratio of DNA: protein in the human chromatins which were subjected to reaction with R3.1. Chang liver and FL cells have a higher ratio of DNA:protein than HeLa chromatin, yet they show lower reactivity than HeLa cells. Other human chromatins (H.Ep-2 & K562), which have similar ratio of DNA:protein as that of HeLa cells, exhibited very little reactivity with R3.1. This suggests no direct correlation between the amount of DNA content and the immunoreactivity of any human chromatin with R3.1 antiserum.

Treatment of HeLa cells with colcemid (Democolcine) resulted in variable immunofluorescent patterns. The differences may reflect the various stages of the cell cycle. This is shown in Figure 4.1.13 and 4.1.14. The fluorescent staining was predominantly found in the whole cell with more intensity at the cytoplasmic boundaries with nuclei (Figure 4.1.13b). A number of cells showed batches of staining distributed either evenly in coarse speckled-like staining (Figure 4.1.13d & e, top) or unevenly in granular pattern (Figure 4.1.13a, centre & top right). The FITC staining indicates the presence of the HeLa antigen(s) during the interphase as well as mitosis. When mitotic cells were cytocentrifuged then fixed and allowed to react with R3.1 (1/100) antiserum, no defined FITC-stained chromosomal structures were identified (Figure 4.1.14).

Table 5: The ratio of DNA:protein of chromatin preparations from human cell lines. The protein content was measured by Bradford's method and the DNA estimated by Burton's procedure.

K562	1:1.9
HeLa	1:1.8
H.Ep-2	1:1.8
Chang liver	1:1.5
FL	1:1.4
KB	1:1.4
HT1080	1:1.4
2M salt extracted chromatin 1:0.214	
2M salt/5M urea extracted chromatin 1:0.097	



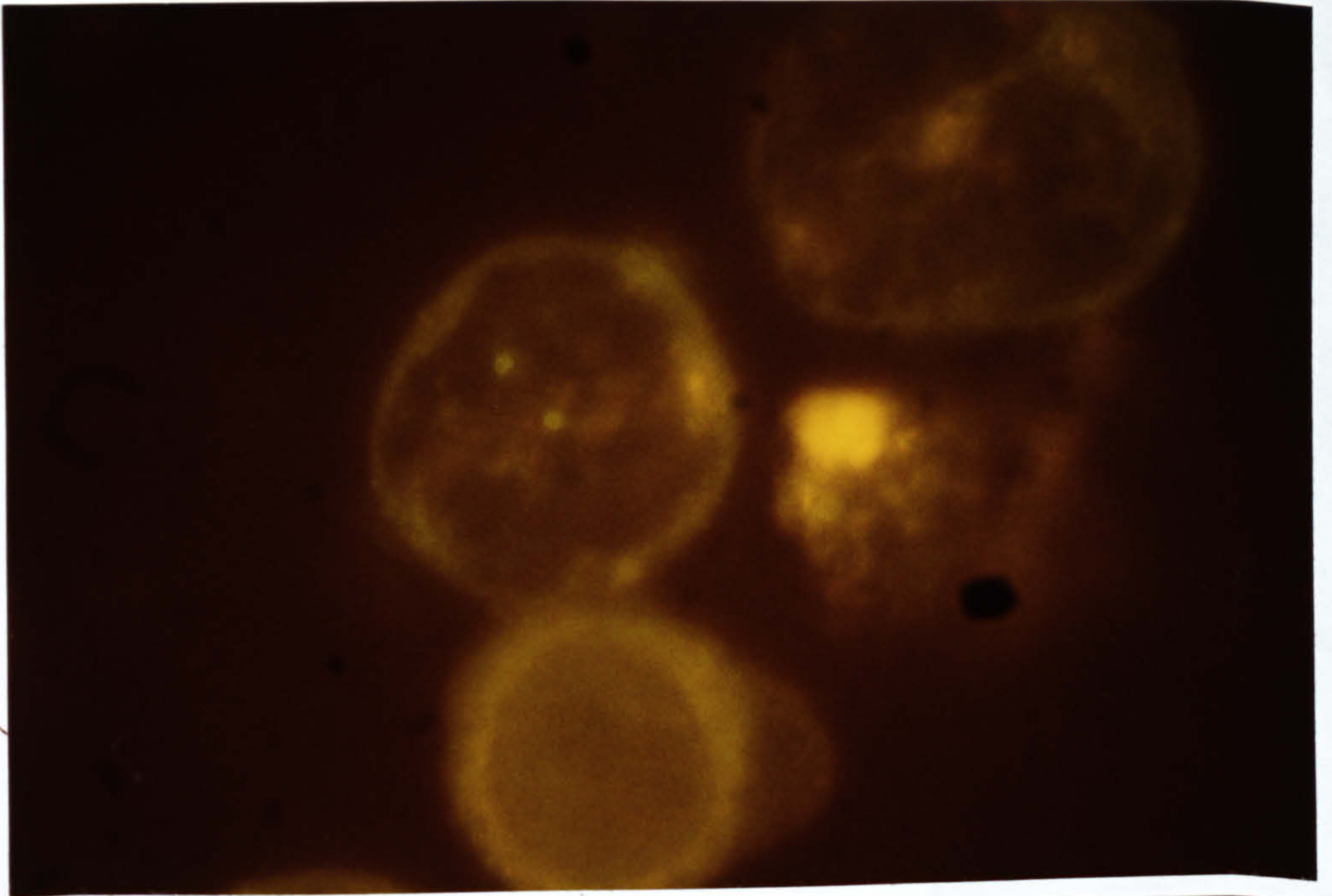
Figure 4.1.13      FITC-staining of HeLa Mitotic Cells Reacted with R3.1  
Antiserum

Indirect immunofluorescent staining of HeLa cells on  
cover slips treated with colcemid for 18 hours, fixed  
and allowed to react with R3.1 antiserum (1/200).

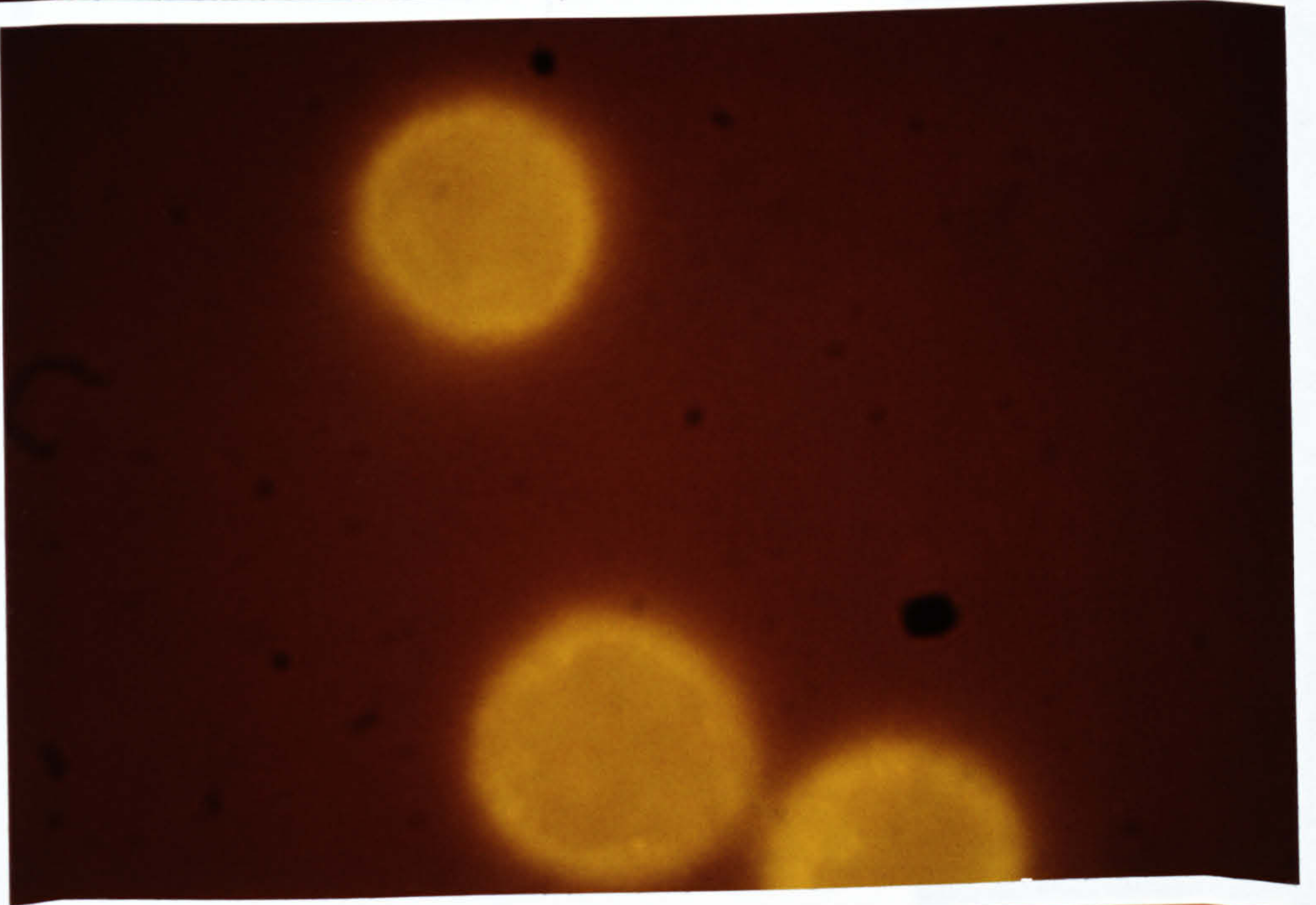
a, b, d :            fluorescent staining (X2000)  
c & e       :           phase contrast of b & d respectively  
                              (X2000)



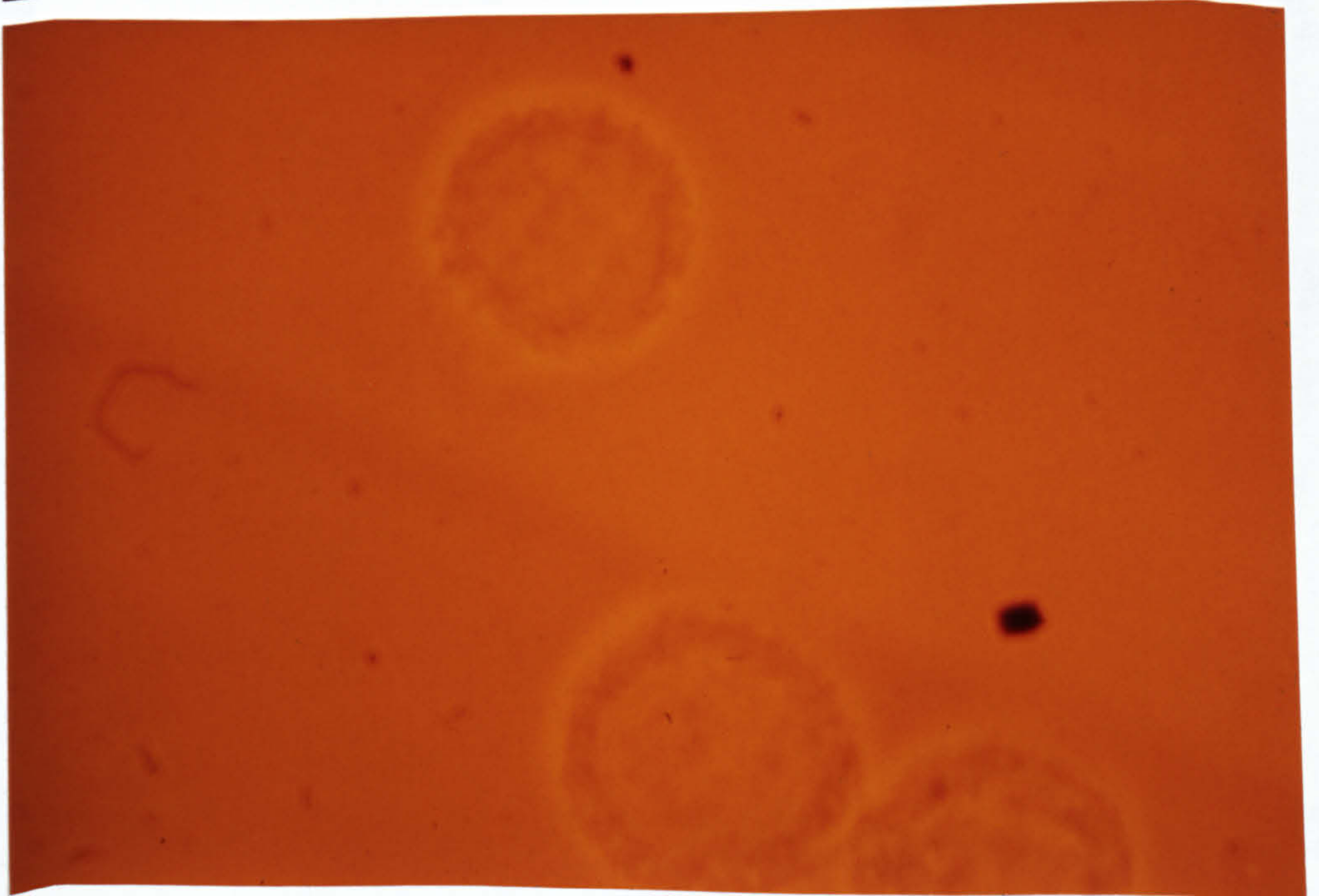
a



b

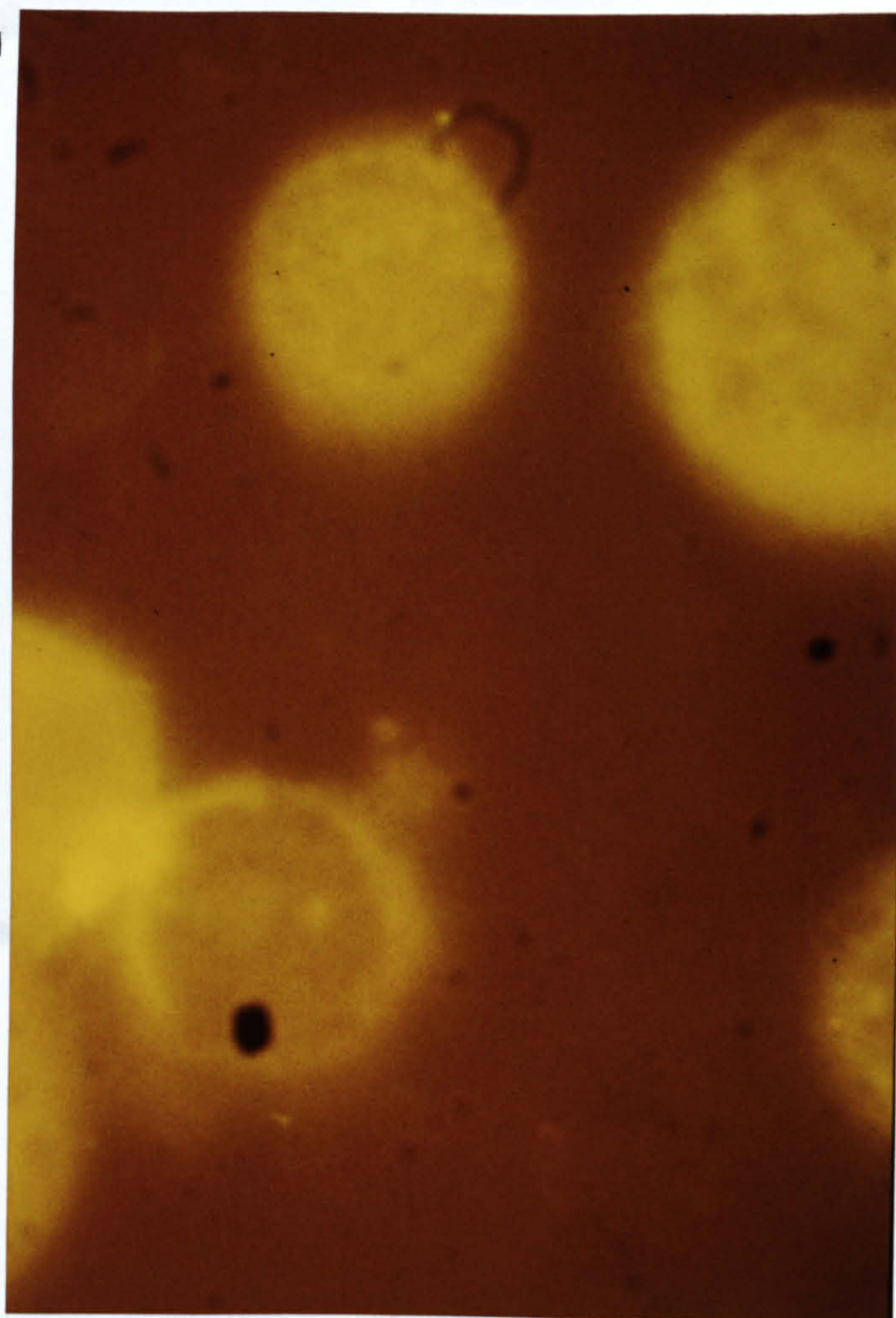


c





d



e

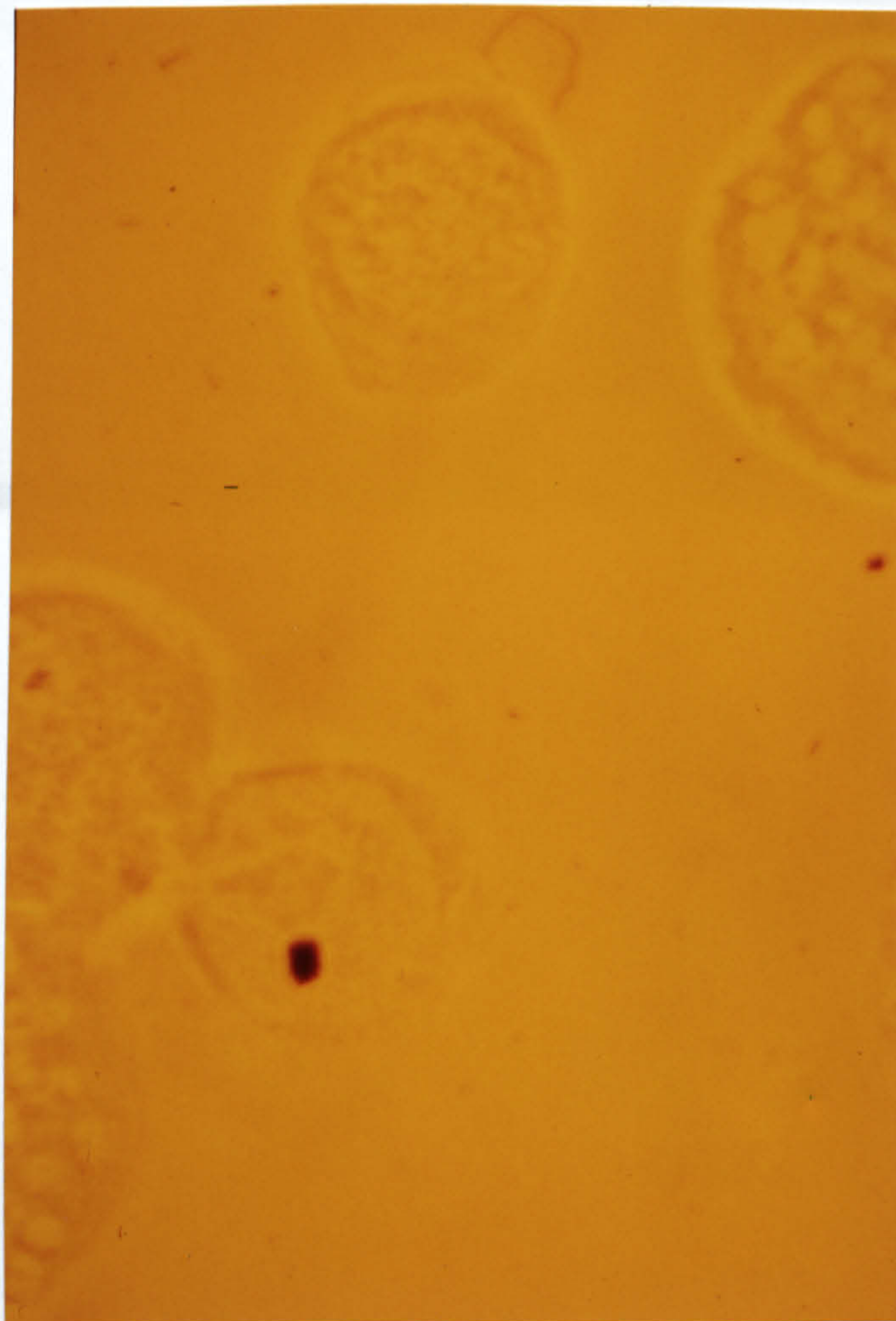




Figure 4.1.14

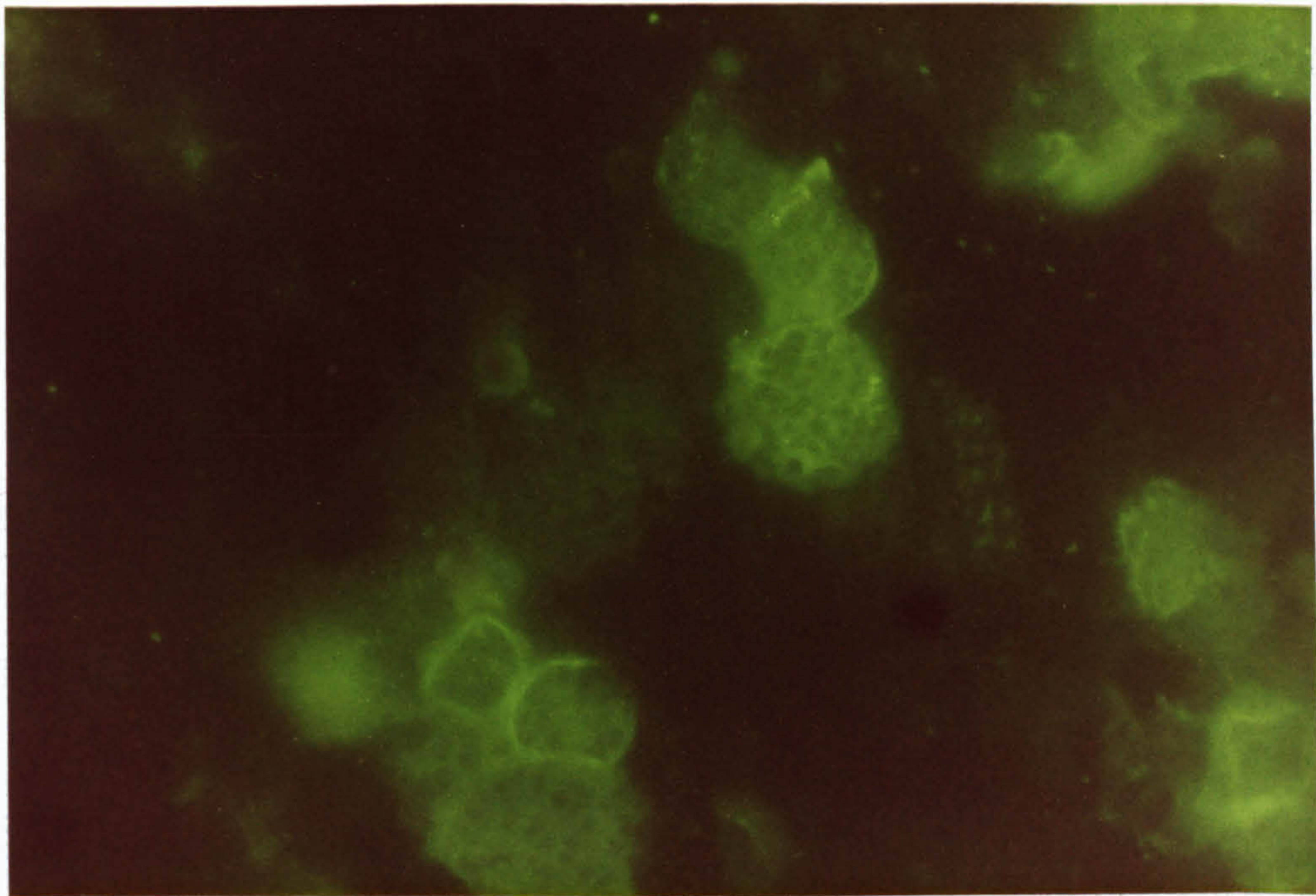
FITC-staining of Mitotic HeLa Cells Cytoentrifuged and  
Reacted with R3.1 Antiserum

Indirect immunofluorescent staining of HeLa cells grown in culture and treated with colcemid for 5h before harvesting. The mitotic cells were cytoentrifuged on slides, fixed then allowed to react with R3.1 (1/100) antiserum.

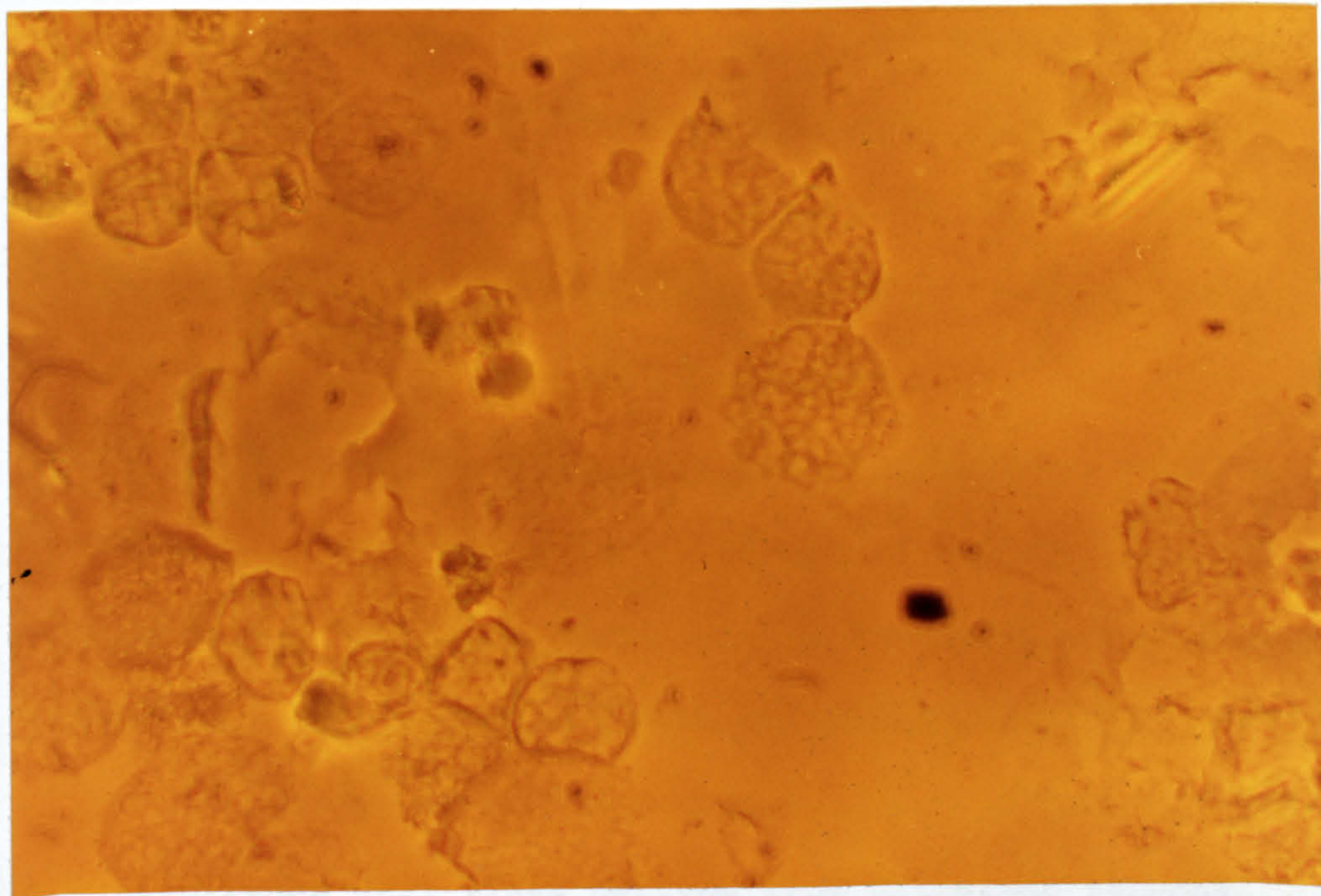
- (a,c) : fluorescent staining (X500 & X2000  
respectively)
- (b) : phase contrast of a (X500)



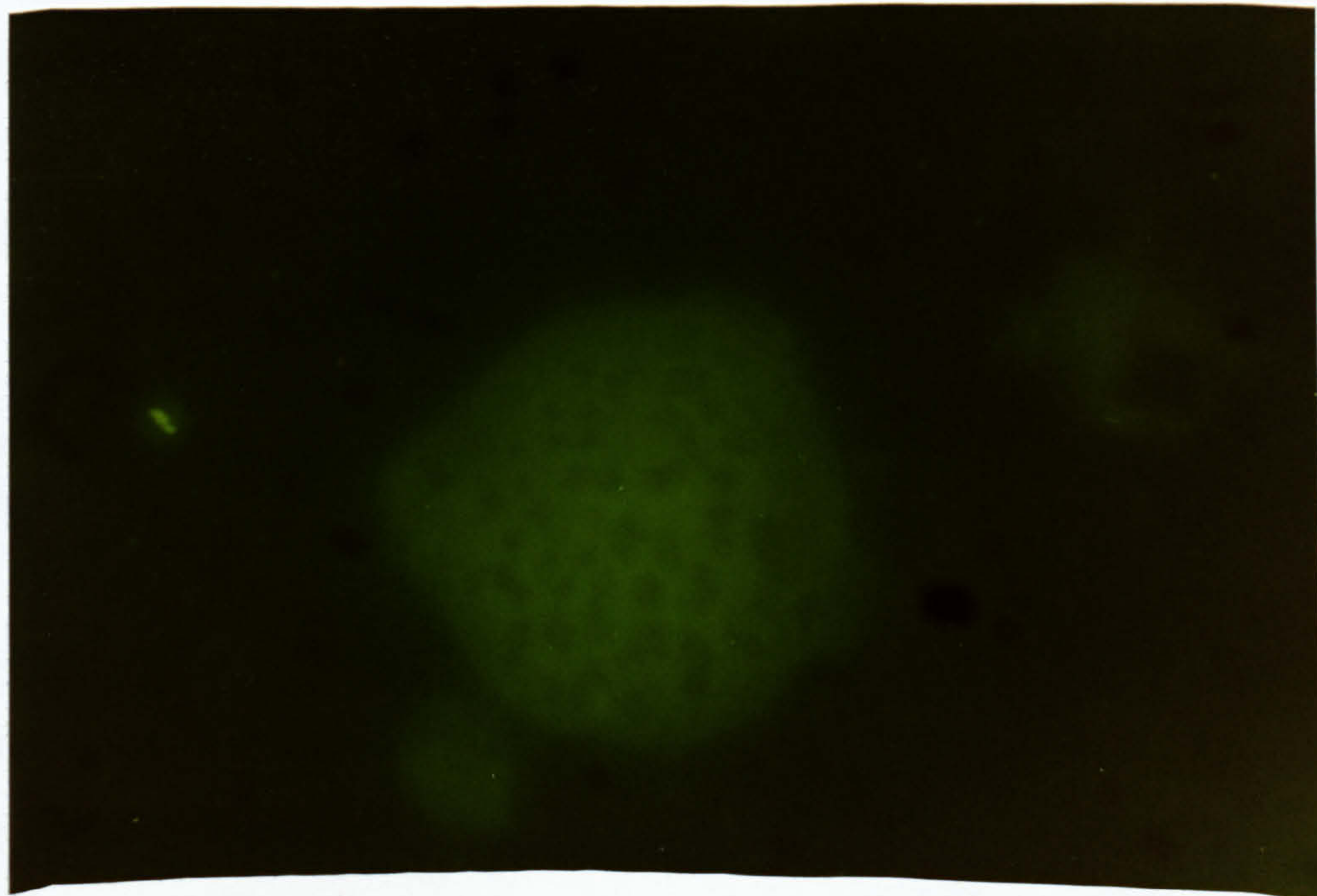
a



b



c





## Discussion

Immunological methods have long been used for the characterisation and detection of nuclear components. The antisera against these components were generally raised in rabbits. Different rabbits respond variably to any given antigen complex. Antisera to HeLa dehistonised nuclear preparations were found by immunocytochemical methods to localise the antigen (or antigen complex) specifically in the nuclei (Campbell et al., 1979; Briggs et al., 1979) or nuclear matrix (Wojtkowiak et al., 1982). Although in these preparations the antigen complex was prepared in a similar way using 2M salt-5M urea extraction, a different pattern of reactivity was observed. The reported antisera obtained against the above 2M salt-5M urea extracted chromatin were raised in white New Zealand rabbits. It is possible that different rabbits may give a different response to this kind of antigen complex. The antiserum R3.1 was raised in a Half Lop rabbit.

The specificity of antisera raised against 2M salt-5M urea extracted fraction has been tested with a number of cell lines (Campbell et al., 1979; Wojtkowiak et al., 1982), but there has been no previous attempt to examine a wider range of tissue-culture established human and non-human cells. In this attempt R3.1 antiserum was allowed to react by C'F and ELISA with human cell lines derived from different tissues (blood, liver, amnion, mouth, skin and larynx). These cells are of epithelial and non-epithelial origin (Table 2a).

In the previous attempts, microcomplement fixation was the only sensitive technique used to examine the specificity of antisera. Examining the specificity pattern of R3.1 antiserum obtained by C'F assay (Figure 4.1.11) implies that R3.1 is cell specific. However, the ELISA technique shows a considerably different reactivity (Figure 4.1.12). Although in the latter technique (ELISA), R3.1 antiserum showed a high reactivity with HeLa chromatin, other human epithelial (Chang liver & FL)

cell lines also exhibited a great deal of immunoreactivity. Therefore, the use of two sensitive techniques which work by two different mechanisms (the microcomplement fixation & ELISA), simultaneously, gives wider scope for looking at the specificity of a given antiserum.

This difference between C'F assay and ELISA has several possible explanations:

1. The plate binding capability of the antigens may vary. ELISA is a very sensitive technique which depends on the binding of the antigens to a solid phase (the ELISA plate). Since there may be a differential capacity of certain proteins to bind to the plate and because ELISA involves several washings which might affect the low affinity binding proteins, an enrichment and increase in the binding of a given component is probable. Moreover, out of the 10 $\mu$ g of chromatin layered per well, an unknown amount of antigen actually binds to the plate (see Figure 4.1.1).
2. Because of the high sensitivity of ELISA, minor antigens, which are common to certain types of tissue culture cells (HeLa, Chang liver & FL epithelial cell lines grown in culture for many years), may be detected, while these antigens are possibly missed by the C'F assay. Added to this is the polyclonality of the antiserum (R3.1) in which antibodies of different abundance and affinity may alter the apparent specificity at various dilutions of antibody and antigen in the two assays.
3. A third possible difference is that the ELISA involves detecting the first antibody by an anti-IgG second antibody, thus the ELISA preferentially selects IgG class of antibodies.

Earlier reports have indicated that the immunogenic components of HeLa dehistonised chromatin reside in the high salt-resistant residual fraction (Campbell et al., 1979). Although only a small amount of protein is tightly bound to the DNA, the immunoreactivity tested by C'F



assay was found to be associated with the residual protein fraction in complex formation with human (homologous) DNA (Dunn et al., 1980). This immunoreactivity was reported to be sensitive to micrococcal nuclease digestion (Campbell et al., 1979; Briggs et al., 1979; Dunn et al., 1980).

Using R3.1 antiserum, it is again possible to demonstrate that a great deal of HeLa chromatin antigenicity is susceptible to micrococcal nuclease digestion (Figures 4.1.2-4.1.4). Such antigenicity that can only be assayed in the presence of two discrete components (DNA & protein) is difficult to analyse for the role played by each separate component. Massive amounts of micrococcal nuclease (7500U/0.3mg DNA/ml) were used for the digestion which drastically reduced the immunoreactivity of chromatin with R3.1 antiserum. About 65-85% of the activity, as monitored by ELISA and C'F assays respectively, was lost (Figures 4.1.2 & 4.1.3). This high loss of immunoreactivity was restored by the addition of HeLa DNA to the digested chromatin.

The earlier reports using antiserum raised in white New Zealand rabbit suggested that only when a large amount of micrococcal nuclease (above 150U/3µg DNA/0.5ml) was used, a considerable degree of immunoreactivity of HeLa chromatin was lost. This was monitored by C'F assay (Campbell et al., 1979). It is possible that the antigen complex which reacts with R3.1 antiserum is different in being highly susceptible to nuclease digestion. Figure 4.1.10 shows that HeLa nuclear lamina-matrix-enriched preparation exhibited only half the activity of the same amount of chromatin preparation. This is believed to be largely due to the use of micrococcal nuclease digestion during the course of the matrix preparation (Section 3.8.7), even though that very small amount of nuclease was involved (for a limited period of time). The amount of micrococcal nuclease used for the lamina preparation was estimated to be approximately 10U/0.3mg DNA/ml.

It is possible that other reasons have contributed collectively to this reduction in immunoreactivity shown in Figure 4.1.10. Among these possibilities are the following:

1. The use of a high concentration of 2-mercaptoethanol to prevent disulfide bridge formation might have reduced the possibility of complex formation (between DNA & protein) and/or may have caused the loss of important proteins essential for the complex component which is in turn necessary for the antigen immunoreactivity. Supporting this idea is the recent finding of Lebkowski and Laemmli (1982) that in the dehistonised chromatin preparation, the addition of 2-mercaptoethanol to the lysis mixture leads to a partial unfolding of the DNA and a selective loss of proteins.
2. Additionally, that the preparation was subjected to a very high salt (3M NaCl) extraction procedure which possibly solubilised some important component(s) of the complex antigen.

Lebkowski and Laemmli (1982) described two forms of histone-depleted HeLa nuclear proteins. Type I protein structure which was obtained by 2M NaCl extraction, is represented by the three nuclear laminar proteins of Mr 60,000-70,000 and high molecular weight residual species. Histone extraction in the presence of 2-mercaptoethanol produced type II structure which comprises almost exclusively the major laminar proteins (Mr 60,000; 68,000 & 70,000) and two minor proteins of Mr 64,000 & 200,000. A laminar protein enriched preparation was also obtained by McKeon et al. (1983), using a slightly different approach.

Wojtkowiak et al. (1982) have raised antisera against 2M salt-5M urea-extracted chromatin fraction from HeLa cells. These sera showed a wide range of cross reactivity not only with other human malignant cell preparations in the immunoblot, but with a number of protein bands in the HeLa chromatin fraction. The antisera also reacted with many bands in the nuclear matrix preparation and many of these proteins were found to

be common to other HeLa subcellular fractions including nucleoli and RNP particles. Detke & Keller (1982) have presented data which suggest that the major chromosome scaffold proteins are also the major proteins of the nucleoskeleton. However, no similarity between the reactive protein bands shown by Wojtkowiak et al. (1982) and the major nucleoskeletal proteins has been noted. Moreover, although these antisera were raised against HeLa antigen preparation similar to that reported by Campbell et al. (1979) and Briggs et al. (1979), no DNA-dependent immunoreactivity was reported.

On the other hand, R3.1 antiserum was elicited against the same dehistonised chromatin fraction (extracted by 2M salt-5M urea) and again its immunoreactivity showed great dependence on the presence of homologous DNA complex formation (Figures 4.1.2 & 4.1.3). Unlike the antisera reported by Wojtkowiak et al. (1982), R3.1 antiserum showed very slight reactivity with proteins on the immunoblot (Figure 4.1.5). Using the sensitive immunoblot technique reported by Towbin et al. (1979), R3.1 antiserum reacted with only two protein bands (Mr 43,000 & 45,000) in the chromatin and dehistonised chromatin fractions (Figure 4.1.5). Since it had been shown that the chromatin antigen which reacted with R3.1 requires complex formation between the DNA and protein(s) (Figures 4.1.2 & 4.1.3), HeLa DNA was added to the blot following the method of Bowen et al. (1980) which was reported to allow considerable DNA binding to the proteins on the blot. The DNA-treated electroblot was then prepared to react with R3.1 antiserum. No major immunoreactivity change was observed except that a protein group of Mr 68,000-70,000 showed marginal reactivity with the antiserum. The failure of additional DNA to produce a major change in the immunoreaction pattern may be because the ability of the DNA to bind to the blot is limited, and not enough is bound to make the necessary conformational alterations on the antigen to make it accessible to antibody reaction. Since it has been observed (see



results section) that SDS treatment of chromatin greatly reduces its immunoreactivity with R3.1 antiserum, another reason why the addition of DNA did not produce a significant change on the protein activity may be due to an irreversible denaturation of the target antigen in the SDS-polyacrylamide gel, so that even after the limited reconstitution with DNA on the blot no extensive renaturation occurred. The third possibility is that the proteins on the blot are restricted by the nature of their binding to the nitrocellulose paper which may not permit an adequate reconstitution with DNA.

There are other points which could also be considered before drawing any conclusion regarding the immunoreactivity of R3.1 on the electroblot.

1. The use of 3% stacking gel and the addition of urea to the gel mixture enhances the protein resolution. This additional modification may possibly trap large molecules of DNA on the top of the gel. It is also possible that some protein molecules, tightly bound to DNA are trapped with it. This is unlikely since the stained gel and the amido-black-stained electroblot showed no protein staining, at the top of both the gel and the blot.

2. It is obvious that large molecules of DNA are unable to enter the high percentage gel. DNA in the concentration of 100µg/ml was added to the blot before its reactivation with R3.1 in an attempt to enhance the antigen immunoreaction. This amount of DNA is not the limiting step for its binding ability, since a lower amount of DNA (10µg/ml) gave a comparatively similar pattern of immunoreactivity. The amount of DNA added to the blot is far more (approximately 10-15 times) than the original DNA content in the chromatin or dehistonised chromatin loaded on the polyacrylamide gel.

Consequently, it is possible to conclude that proteins 68,000 and 70,000 are part of the HeLa immunogen complex which requires DNA

binding for its immunoreactivity with R3.1 antiserum. Based on the immunofluorescent staining (Figure 4.1.6) and the results in Figure 4.1.10, which show a considerable amount of reactivity lying within the nuclear lamina-enriched HeLa preparation (predominantly comprises the 60,000; 68,000 & 70,000 proteins as reported by McKeon et al., 1983), it may be suggested that part of the antigen complex is associated with nuclear lamina proteins or proteins which constitute a component of the nuclear membrane. The former possibility (i.e. association with lamina proteins) is more likely. Supporting evidence for this is the different distribution of fluorescent reactivity, after treatment with colcemid, which indicates the presence of the antigen at various stages of the cell cycle (Figures 4.1.6, & 4.1.13 & 4.1.14). This could be correlated with Detke & Keller's (1982) report that the lamina proteins are dispersed throughout the cell cycle.

The high titre of immunoreactivity of R3.1 antiserum towards HeLa chromatin (Figures 4.1.1, 4.1.11 & 4.1.12) contrasts strongly with the poor reaction seen on the immunoblot (although the technique is very sensitive) and it is important to clarify this discrepancy. It is likely that the major antigenic reactivity can not be visualised on the immunoblot. This speculation can lead to a more feasible conclusion. The major antigen complex which is reactive with R3.1 antiserum may be a large complex of delicately (and maybe specifically) associated DNA with protein. It is also possible that more than one protein is participating in this complex immunoreactivity, and that upon separating the protein components by electrophoresis, no significant restoration of activity on the immunoblot, even after the addition of DNA, could be achieved. It is likely that the major immunoreactivity of this complex is highly dependent on this delicate and balanced association between the two discrete components (DNA & protein). The immunogen complex is susceptible to disorganisation by the effect of SDS treatment, electrophoresis and DNA digestion.

#### 4.2 Results Obtained Using R2.1 Antiserum

R2.1 antiserum was raised in a white English rabbit immunised with 2M salt-extracted HeLa chromatin given as two (150µg DNA) subcutaneous injections ten days apart and one intravenous booster six days before bleeding the animal.

The antiserum showed a high titre on reaction with HeLa chromatin. The immunoreaction was demonstrated by C'F assay (Figure 4.2.1) and ELISA (Figure 4.2.2). A dilution as high as 1/1200-1/1600 was still enough to show a considerable amount of immunoreactivity with the HeLa antigen.

When R2.1 antiserum was allowed to react with other chromatins of human and non-human origin, different patterns of reactivity were observed. In C'F assay, two human epithelial cell lines, KB and Chang liver chromatins showed little C'F activity (20%) when reacted with R2.1 antiserum at a dilution of 1/300 (Figure 4.2.1a). Similar results were found by ELISA, where KB and Chang liver chromatins showed just below 20% of the activity of HeLa chromatin at an antiserum dilution of 1/200-1/400 (Figure 4.2.2a). However, a non-epithelial human cell line (K562) showed high reactivity with R2.1 at serum concentration of 1/200-1/300 in both the C'F and ELISA techniques.

Other human chromatins from cell lines such as the laryngeal carcinoma (H.Ep-2) and the fibrosarcoma (HT1080) also exhibited little (30%) immunoreactivity in comparison with HeLa chromatin, when tested by ELISA (Figure 4.2.2a).

In contrast to the human chromatins, chromatins from non-human cell lines (BSC-1, L929 & NS1) showed no significant immunoreactivity (less than 10%) in comparison to HeLa activity in both the C'F and ELISA techniques (Figures 4.2.1b & 4.2.2b). These cell lines were green African monkey kidney, mouse fibroblast and mouse myeloma respectively.



Figure 4.2.1a

C'F Assay of R2.1 with Human Chromatins

Complement fixation assay of various human chromatins (20µg of protein) fixed with different concentrations of R2.1 antiserum. Antibody and antigen controls were subtracted.

- HeLa
- △ K562
- Chang liver
- ▽ KB

Antibody control at 1/300 dilution showed a 5% C'F while it was only 1% at 1/1200 dilution. Antigen controls were as follows:

0% C'F : HeLa, K562  
1% C'F : Chang liver  
4% C'F : KB

**Figure 4.2.1a**

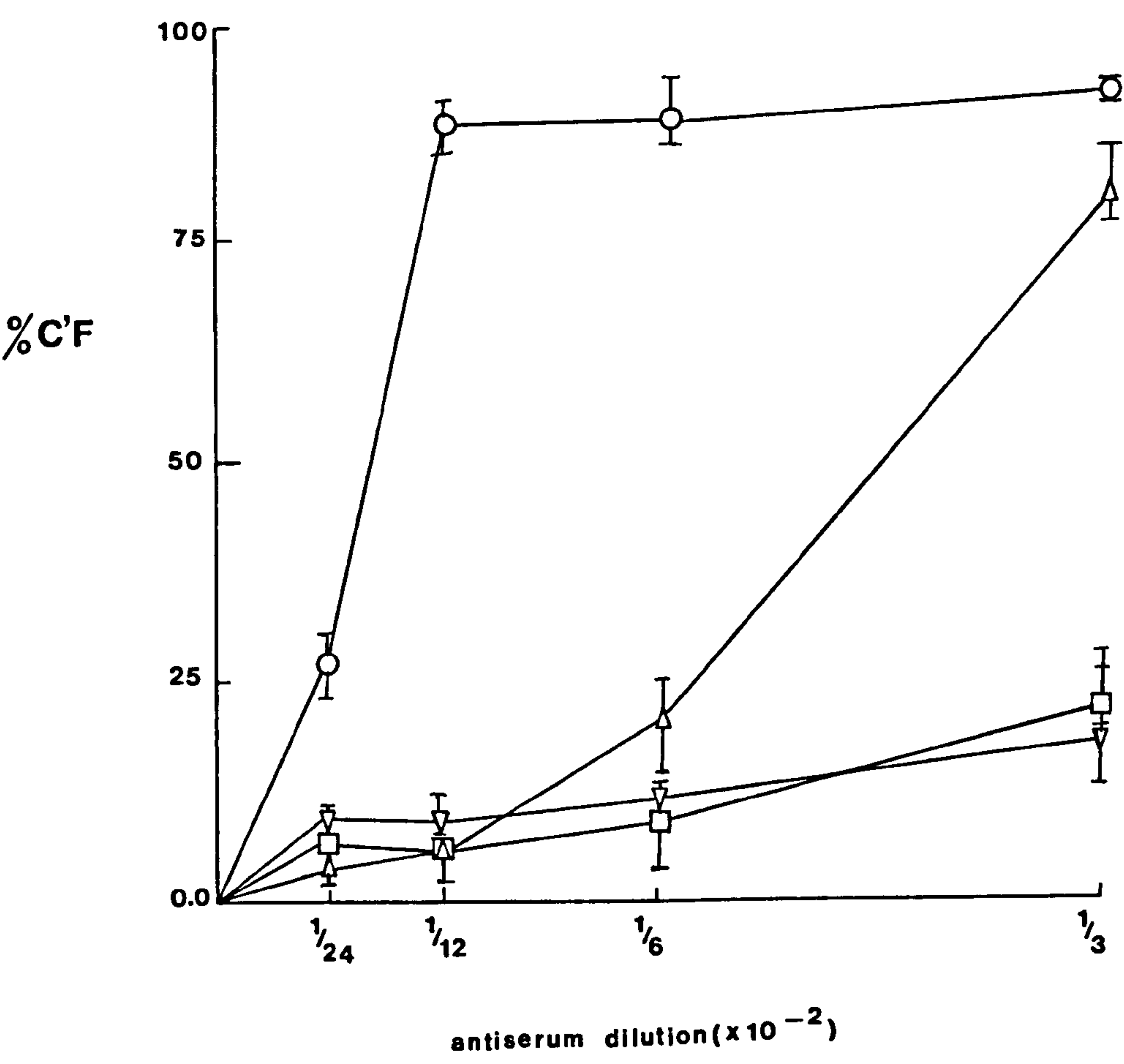


Figure 4.2.1b

C'F Assay of R2.1 with Non-human Chromatins

Complement fixation assay of HeLa chromatin compared with various non-human chromatins (20µg of protein) fixed with different concentrations of R2.1 antiserum. Antibody and antigen controls were subtracted.

- HeLa
- ▲ L929
- BSC-1
- ▽ NS1

Antibody control at 1/300 dilution showed a 5% C'F while it was only 1% at 1/1200 dilution. Antigen controls were as follows:

- 0% : HeLa, NS1
- 1% : BSC-1, L929



Figure 4.2.1b

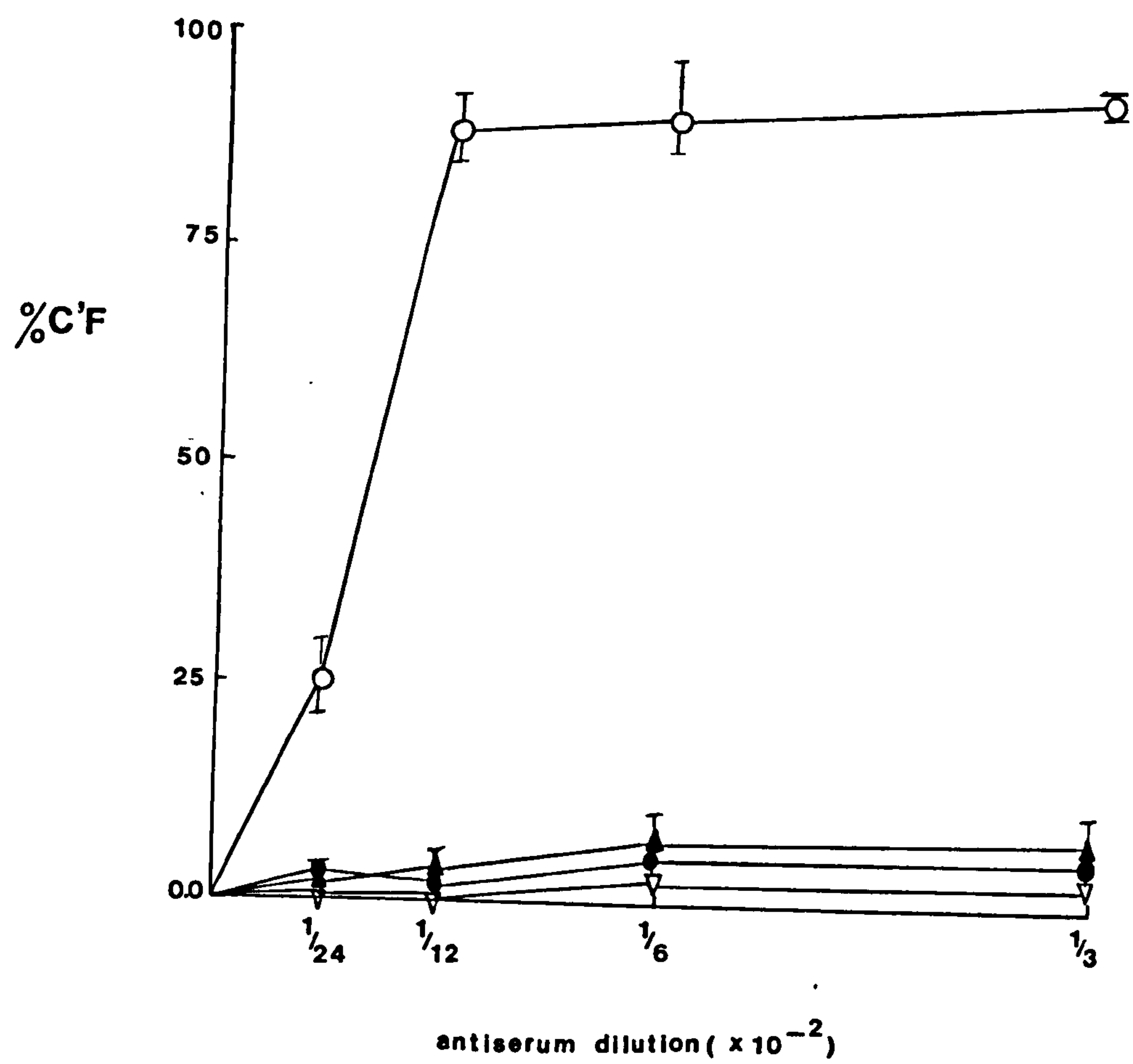


Figure 4.2.2a

ELISA of R2.1 with Human Chromatin

Enzyme linked immunoassay of different concentrations of R2.1 antiserum reacted with human chromatin (10µg of protein layered in each well) immobilised on an ELISA plate. Antigen and antibody controls were subtracted.

- HeLa
- HT1080
- ∇ KB
- Δ K562
- Chang liver
- H.Ep-2

Antibody control was highest at 1/200 dilution (0.08 A<sub>492</sub> unit) while antigens controls were as follows:

HeLa	:	0.126 A <sub>429</sub> unit
HT1080	:	0.102 A <sub>492</sub> unit
KB	:	0.101 A <sub>492</sub> unit
K562	:	0.105 A <sub>429</sub> unit
Chang liver	:	0.107 A <sub>492</sub> unit
H.Ep-2	:	0.129 A <sub>492</sub> unit

**Figure 4.2.2a**

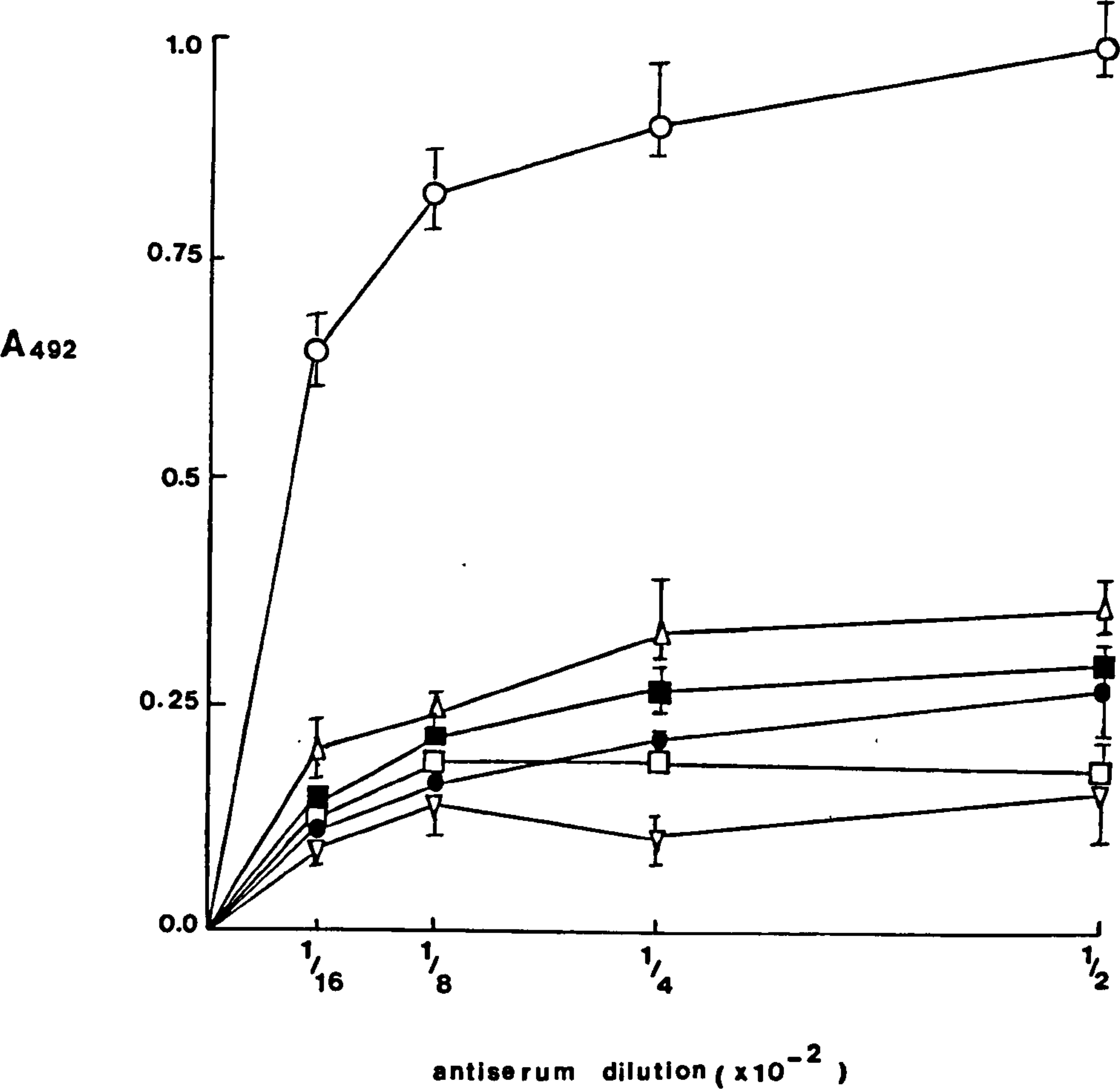




Figure 4.2.2b

ELISA of R2.1 with Non-human Chromatins

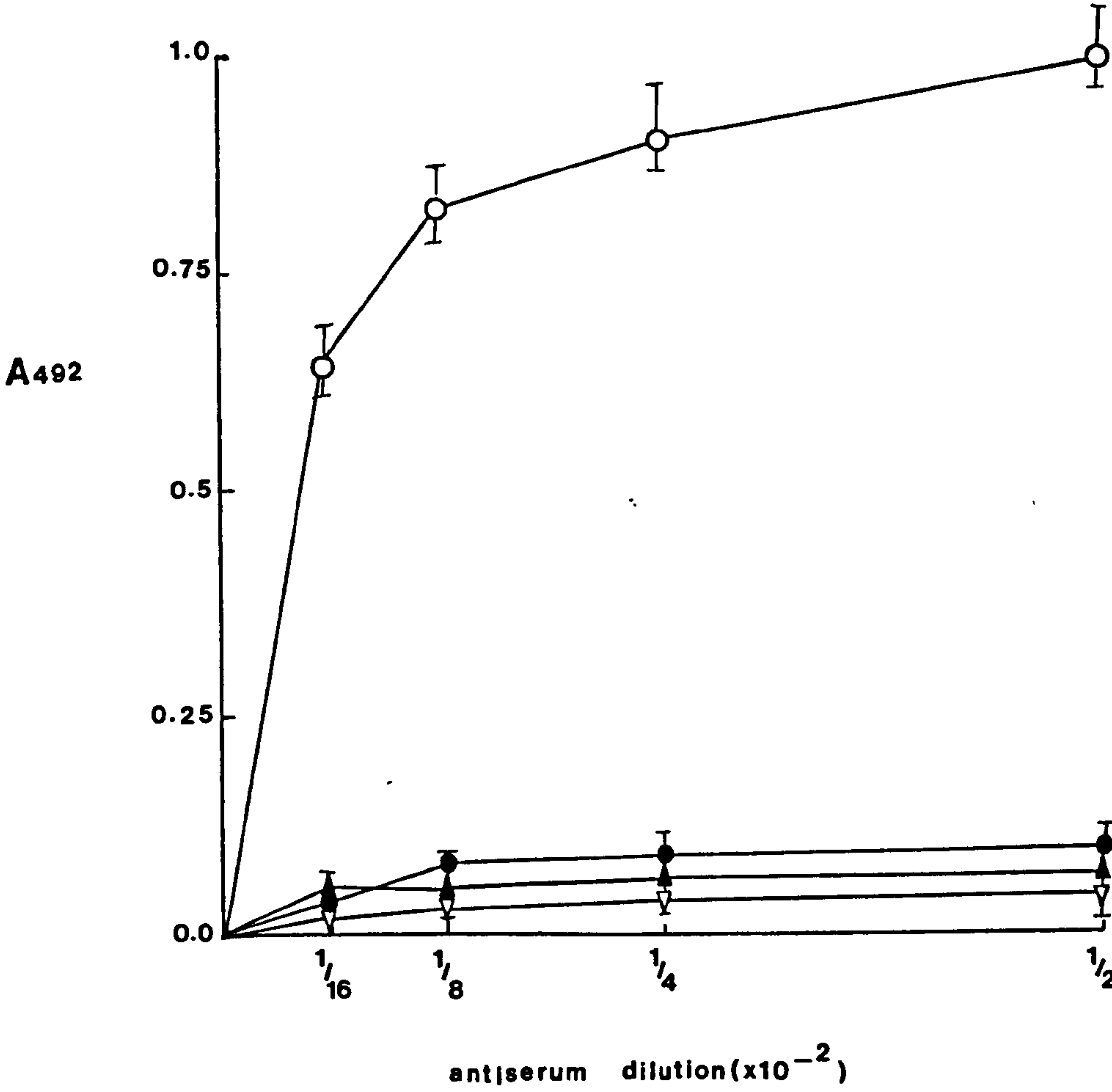
Enzyme linked immunosorbent assay of different concentrations of R2.1 antiserum reacted with HeLa and non-human chromatins (10µg of protein layered in each well) immobilised on an ELISA plate. Antibody and antigen controls were subtracted.

- HeLa
- ▲ L929
- BSC-1
- ▽ NS1

Antigen controls were found to be:

HeLa	:	0.126 A <sub>492</sub> unit
L929	:	0.111 A <sub>492</sub> unit
BSC-1	:	0.130 A <sub>492</sub> unit
NS1	:	0.120 A <sub>492</sub> unit

Figure 4.2.2b



To demonstrate whether R2.1 reactivity with HeLa chromatin is dependent on DNA:protein complex formation or not, the chromatin was digested with large amount of micrococcal nuclease (2500U/0.1mg/ml). The nuclease digested chromatin was then allowed to react with R2.1 antiserum using the ELISA. About 70% of the chromatin immunoreactivity with R2.1 was retained after nuclease digestion (Figure 4.2.3). The lost immunoreactivity was almost restored on reconstituting the digested chromatin with HeLa DNA alone (Section 3.8.2). The HeLa DNA alone exhibited very low reaction (10%) compared with the chromatin reactivity (Figure 4.2.3). This suggests that approximately a third of HeLa chromatin reactivity with R2.1 antiserum requires the presence of HeLa DNA. This part of the immunoreactivity does not lie within the DNA alone.

In order to determine the protein antigen(s) of HeLa chromatin and dehistonised chromatin, these fractions were run on an SDS-gel and then transferred onto nitrocellulose paper and allowed to react with R2.1 antiserum (1/250) using the immunoperoxidase method described in Section 3.5.4.1. Figure 4.2.4 shows a typical result. Both the chromatin fraction and the 2M salt-extracted chromatin (lanes 6 & 7) showed an intense reaction corresponding to a range of approximate molecular weights of 42,000-52,000.

In an attempt to find out whether preincubating the protein blot with HeLa DNA has any effect on the pattern of the immunologically reactive proteins or not, one strip of protein-containing nitrocellulose paper was incubated with DNA as described in Section 3.6.3.5 then allowed to react with R2.1 antiserum. Chromatin proteins (lanes 8 & 10) show the same high colour intensity indicating that the reactive proteins are of molecular weights 42,000-52,000. Moreover, the intense and thick band of the 2M salt-extracted fraction (lane 9) extends further to



Figure 4.2.3

ELISA of R2.1 with Nuclease-digested and -undigested  
HeLa Chromatins

Enzyme linked immunoassay of R2.1 antiserum (1/250) allowed to react with different concentrations of HeLa antigen immobilised on an ELISA plate. Antibody and antigen controls were subtracted. (Chromatin measured as total protein content and HeLa DNA estimated as total DNA content).

- Undigested HeLa chromatin
- Micrococcal nuclease-digested HeLa chromatin  
reconstituted with HeLa DNA
- Δ Micrococcal nuclease-digested HeLa chromatin
- ∇ HeLa DNA alone

The average antibody control was 0.1 while antigen controls varied slightly with the amount of antigen plated and the highest was found at about 0.17 A<sub>492</sub> unit for the undigested chromatin at 2.25μg concentration.

**Figure 4.2.3**

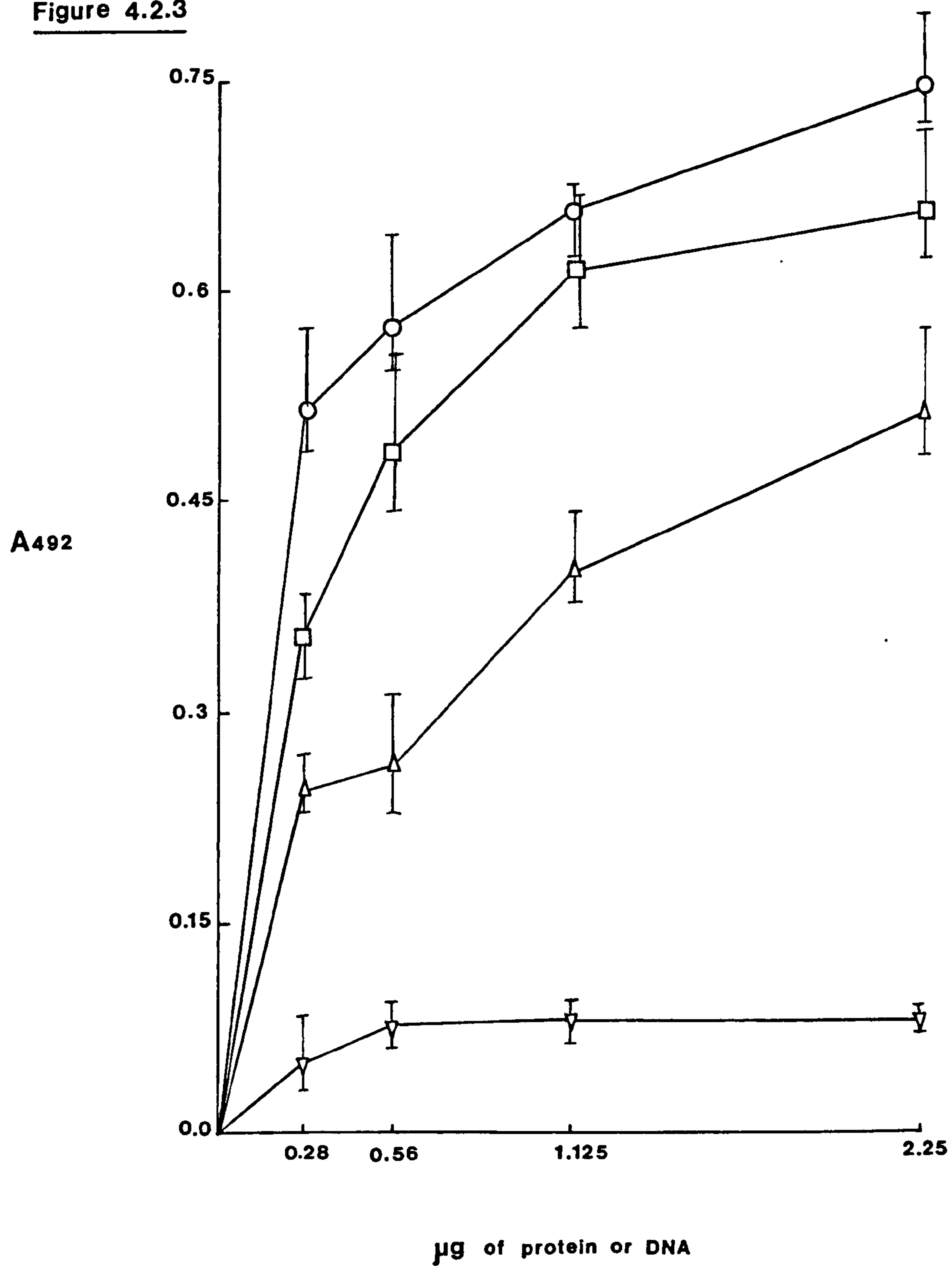




Figure 4.2.4

Protein Blot of HeLa Chromatin Reacted with R2.1 and  
Normal Rabbit Sera

Protein immunoblot of R2.1 antiserum (strips C and D) and normal rabbit serum (strip A) reacted, at a dilution of 1/250, with chromatin (lanes 1,6,8 and 10) and 2M salt-extracted chromatin (lanes 2,7 and 9) preparations from HeLa cells visualised by using the immunoperoxidase method. Lanes 3 and 5 are the amido-black stained proteins corresponding to chromatin and dehistonised chromatin respectively. Lane 4 is the molecular weight marker (from top: phosphorylase b 94,000; bovine serum albumin 67,000; ovalbumin 43,000; carbonic anhydrase 30,000; soybean trypsin inhibitor 20,100;  $\alpha$ -lactalbumin 14,400). The proteins were analysed in a 10% Laemmli polyacrylamide gel containing 0.5M urea. Strip D has been incubated with HeLa DNA prior to its reaction with R2.1.

Although the core histone proteins were well separated (lane 3), H1 histone was not found as intense (in our chromatin preparation) as any individual core histones.



...a range of ...

...and again ...

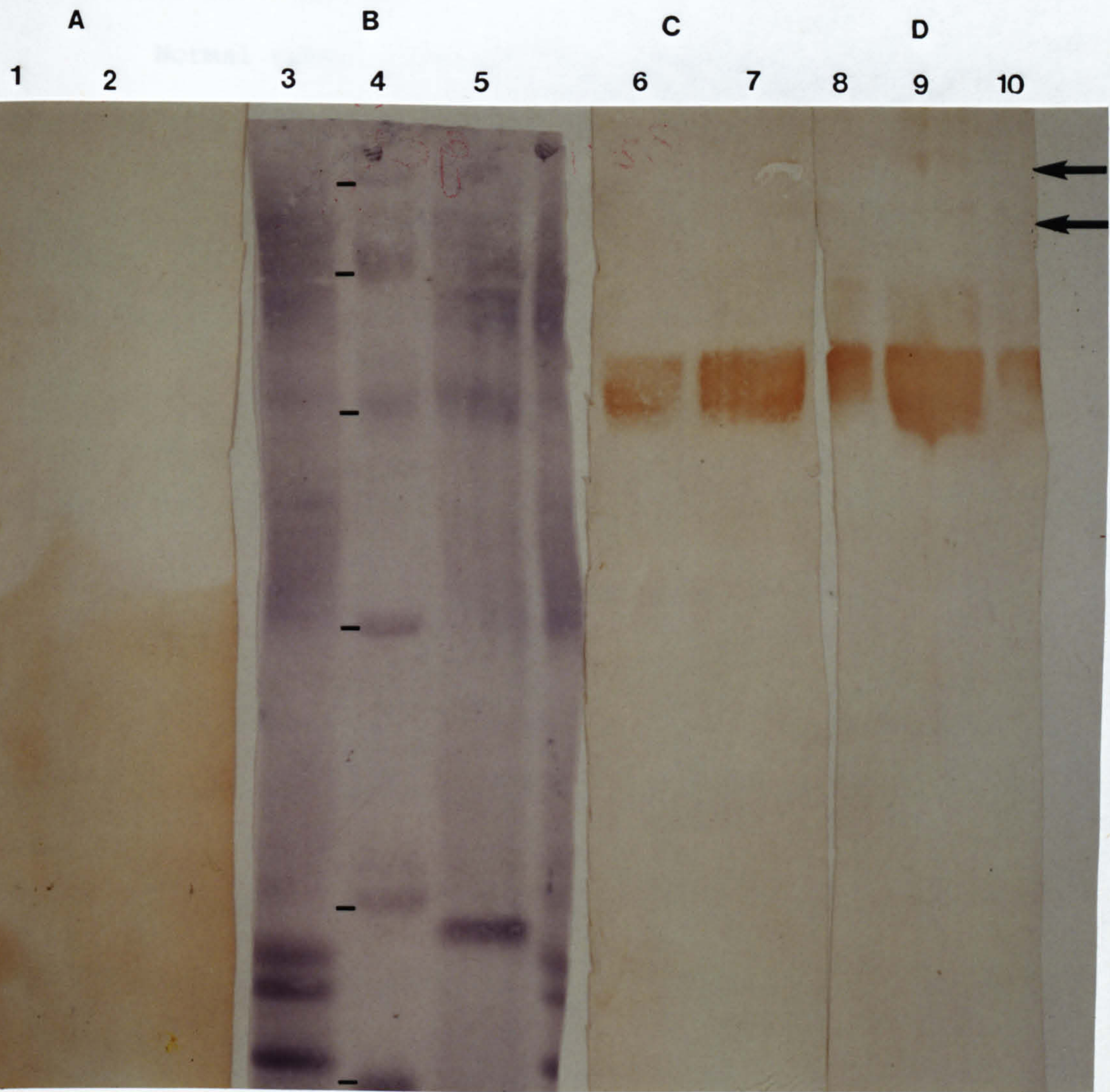
...of 52,000-54,000 ...

...fraction ...

...body reaction ...

...at approximately ...

...treated ...



...and ...

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In order to ...

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encompass a range of 40,000-52,000 of molecular weight. In addition, a faint and again unresolved thick band corresponding to a molecular weight range of 52,000-64,000 was visible in both chromatin and dehistonised chromatin fractions only on pretreating the blot with DNA prior to the antibody reaction. Another, very faint but single, protein bands can be seen at approximate molecular weights of 80,000 and above 100,000 in the DNA treated blot (arrows).

Normal rabbit serum (1/250) again failed to show any reactivity with HeLa chromatin and dehistonised chromatin.

Examination of the amido-black stained blot shows that both chromatin (lane 3) and dehistonised chromatin (lane 5) have one detectable band at the range of Mr 43,000-45,000. No other band at the range of Mr 40,000-52,000 could be seen. This may indicate the presence of amido-black-undetected proteins above and below the 43,000-45,000 protein band(s). While these proteins are not detectable by amido-black staining, they are readily observed by immunoblotting. This exemplifies the usefulness and efficiency of the sensitive immunoblotting technique.

Indirect immunofluorescence was employed to determine the subcellular localisation of the HeLa antigen. HeLa cells were grown on coverslips, washed, lysed, fixed and allowed to react with R2.1 antiserum (1/100) then FITC-conjugated second antibody. The immunofluorescent stained-cells were inspected under the fluorescent microscope. Figure 4.2.5 demonstrates that the immunoreactivity is distributed in both nuclei and cytoplasm. The fluorescent staining is more intense in nuclei with negatively stained nucleoli (Figure 4.2.5a-e). Filamentous structures extending from the cytoplasmic periphery (Figure 4.2.5f & g) could also be seen using high magnification.

In order to examine whether the cytoplasmic staining was of the cytokeratin type or not, the FITC technique was employed with the aim of preserving the structure of the keratin filaments. The method is



Figure 4.2.5

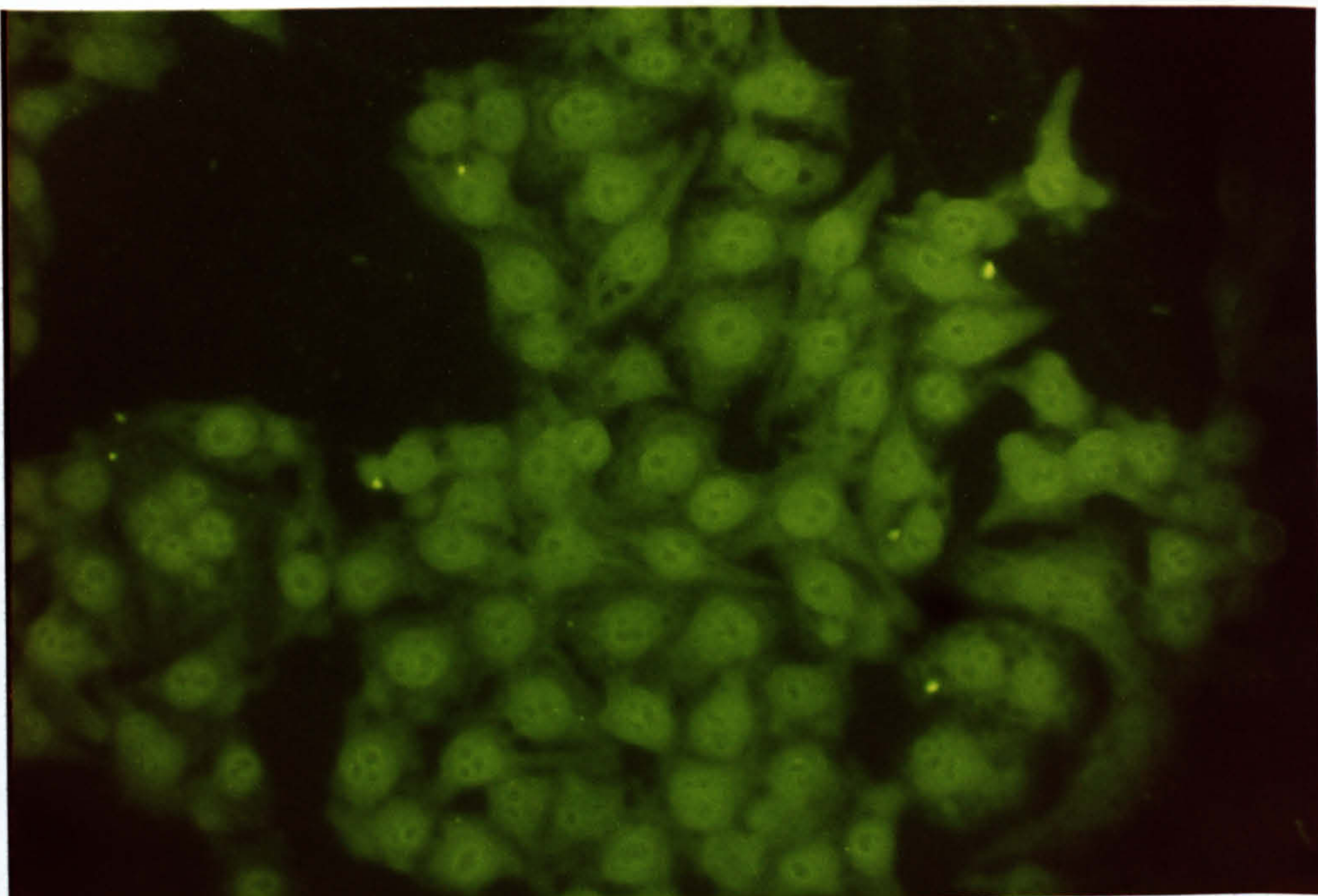
FITC-staining of HeLa Cells Reacted with R2.1

Indirect immunofluorescent staining of HeLa cells reacted with R2.1 (1/100) antiserum. The cells were grown on coverslips and treated as described in section 3.7.4.

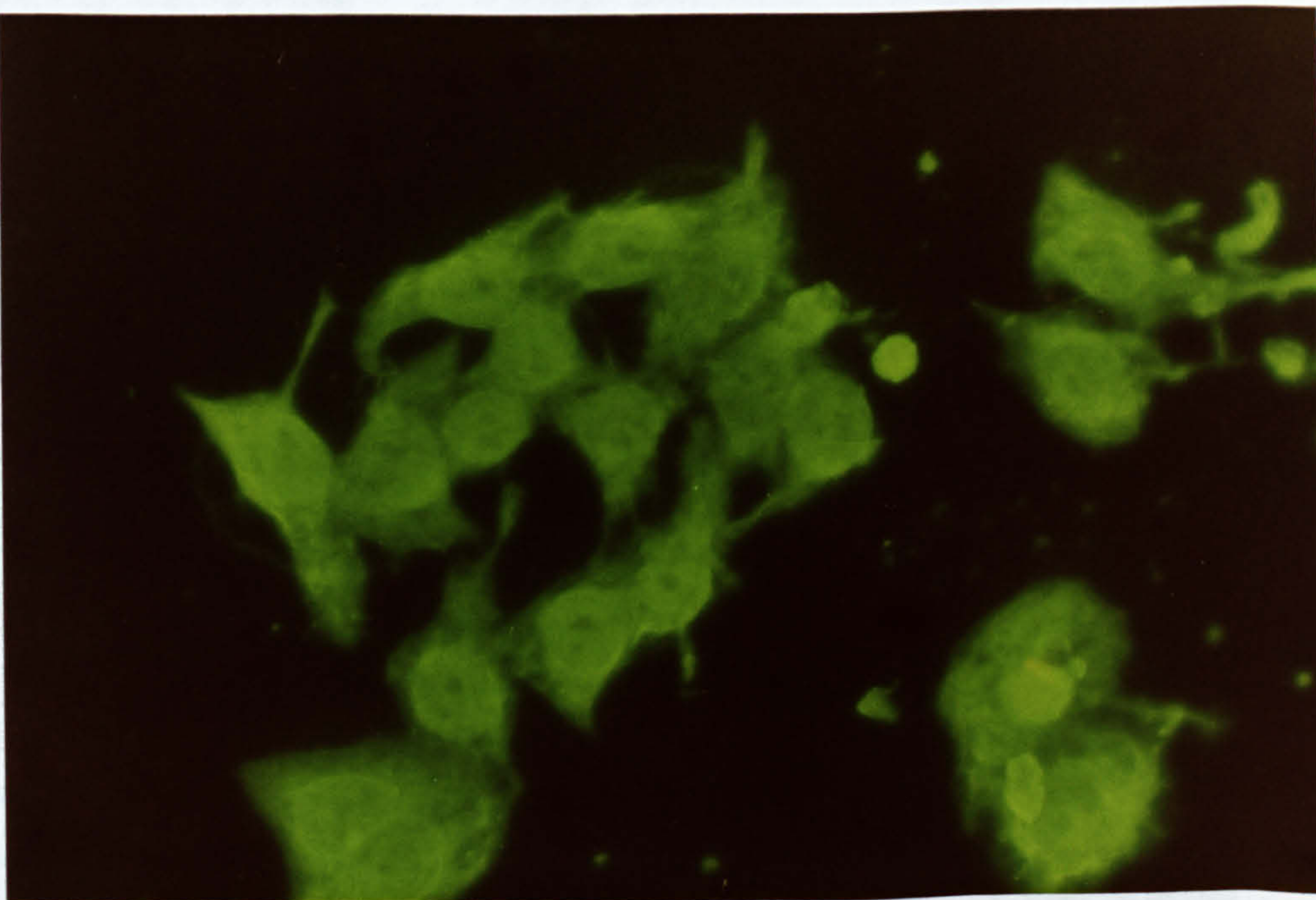
- |         |                                                |
|---------|------------------------------------------------|
| (a)     | X310                                           |
| (b & c) | X500                                           |
| (d & e) | X2000                                          |
| (f & g) | X2000 with reference to filamentous structures |



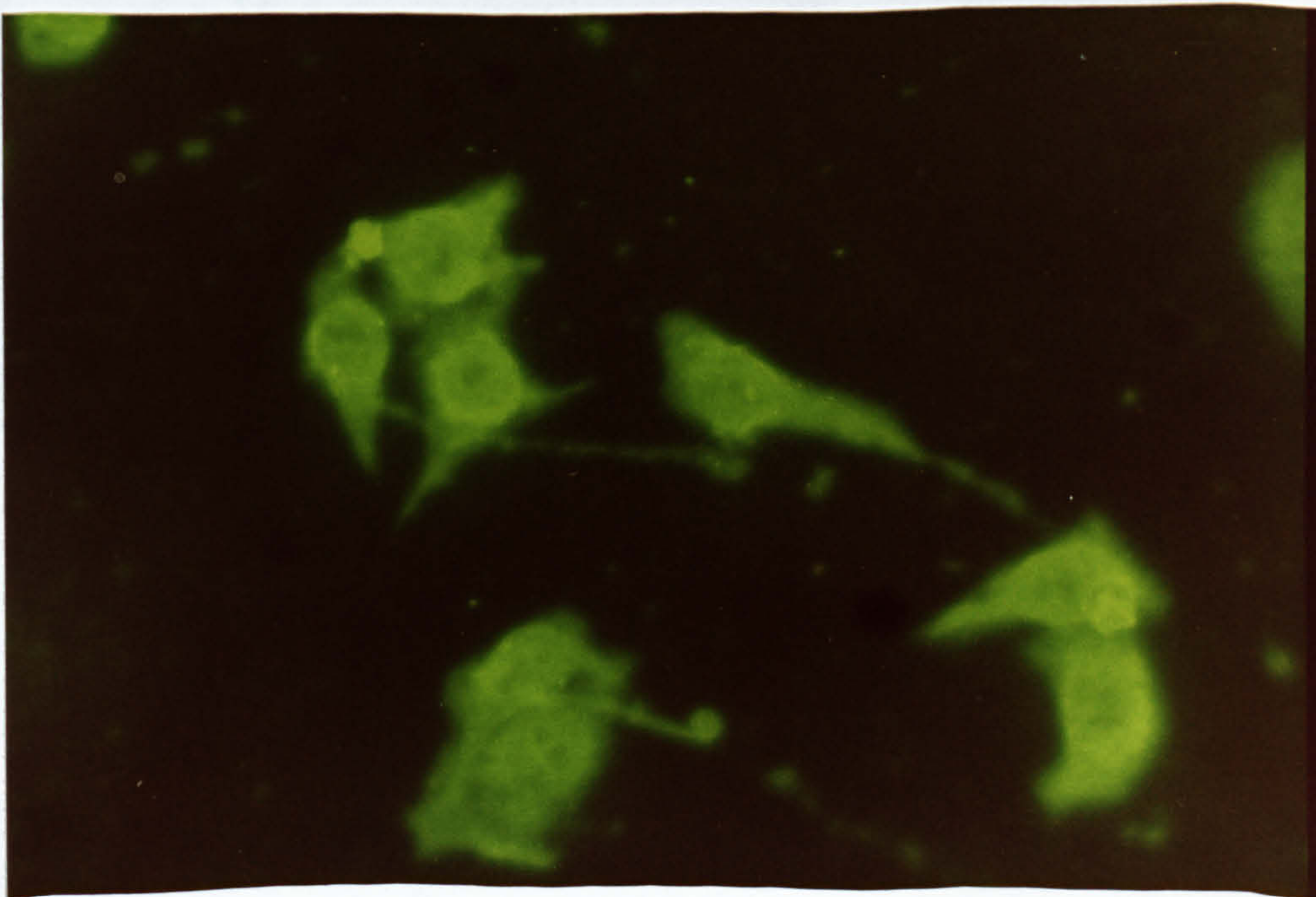
a



b

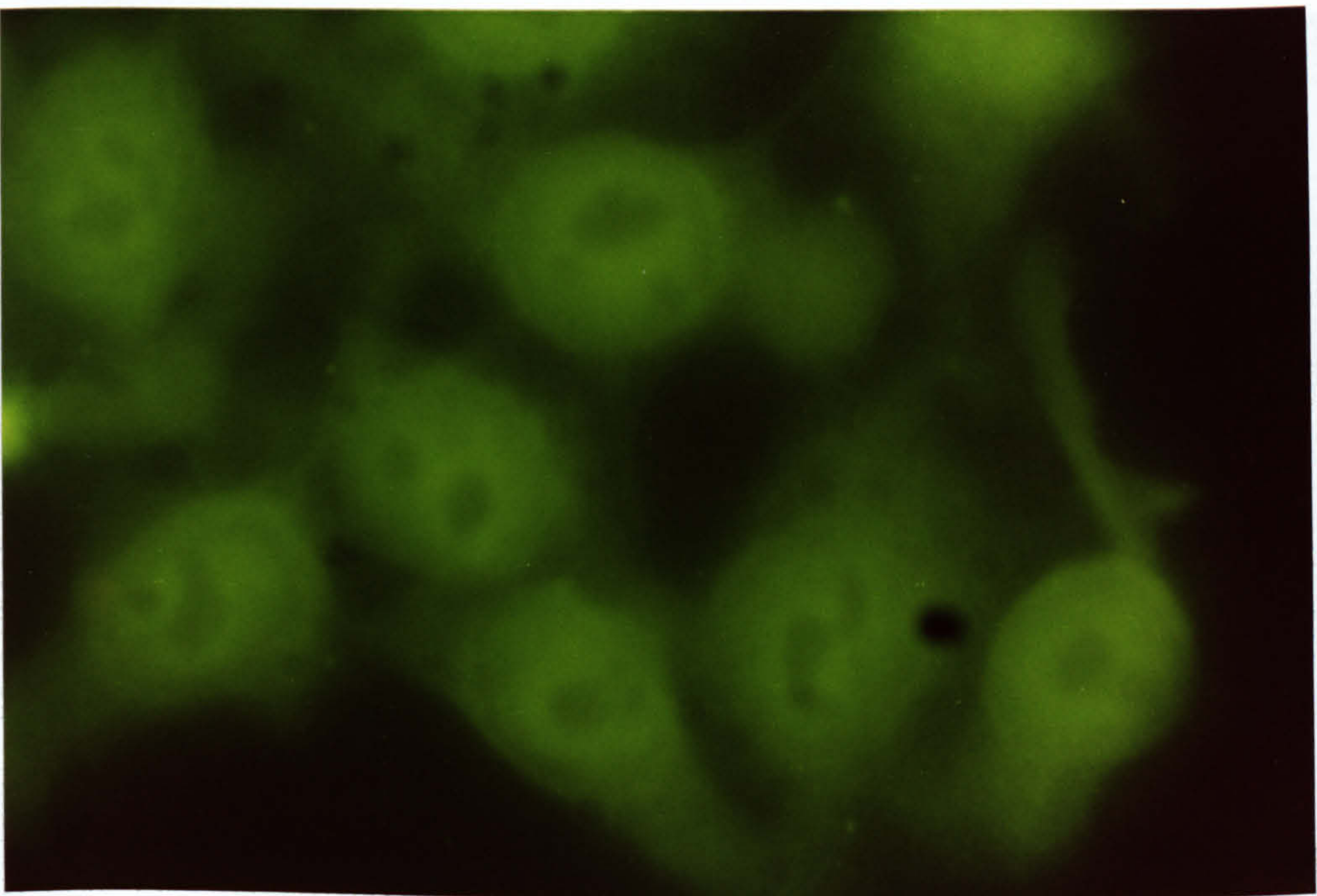


c

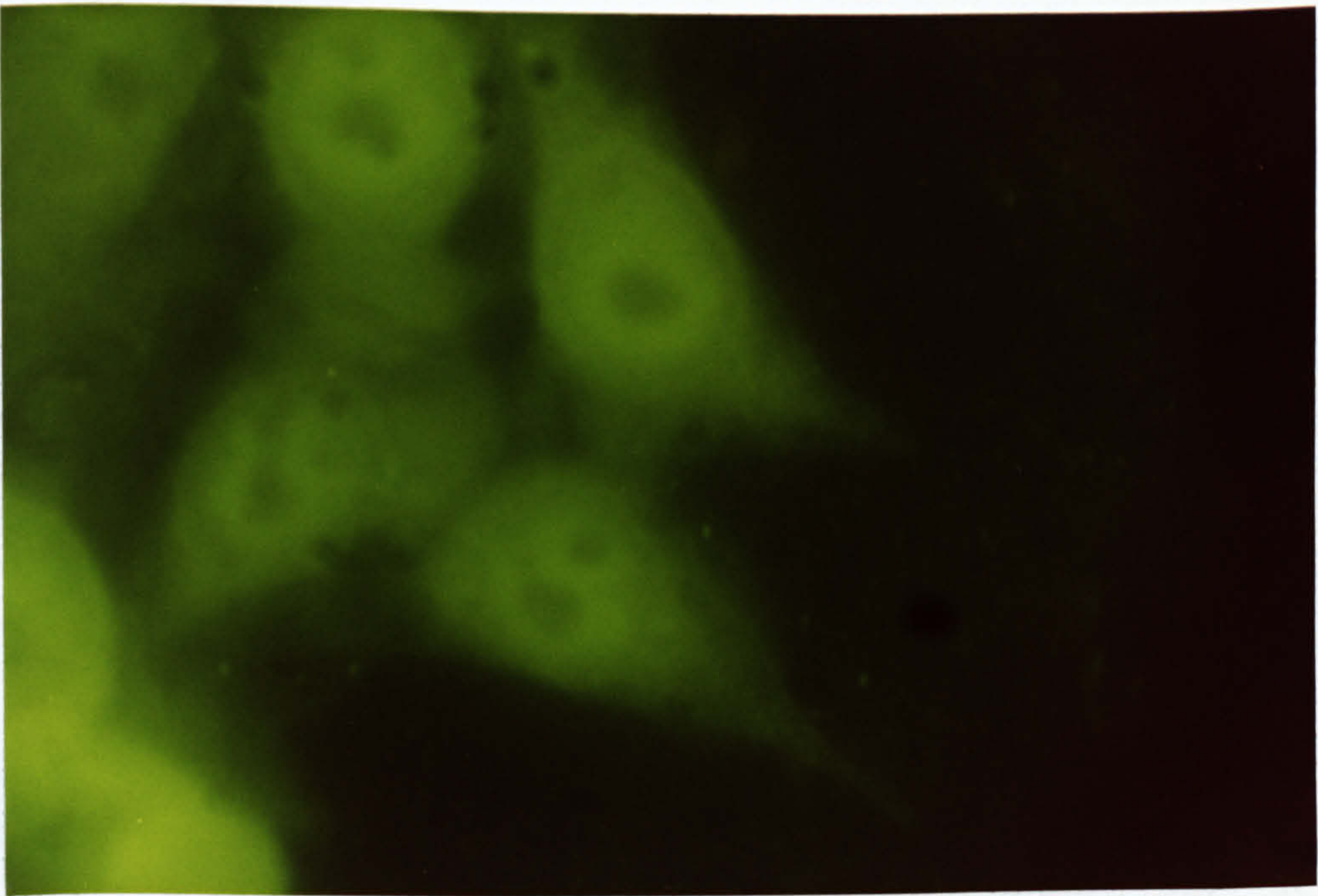




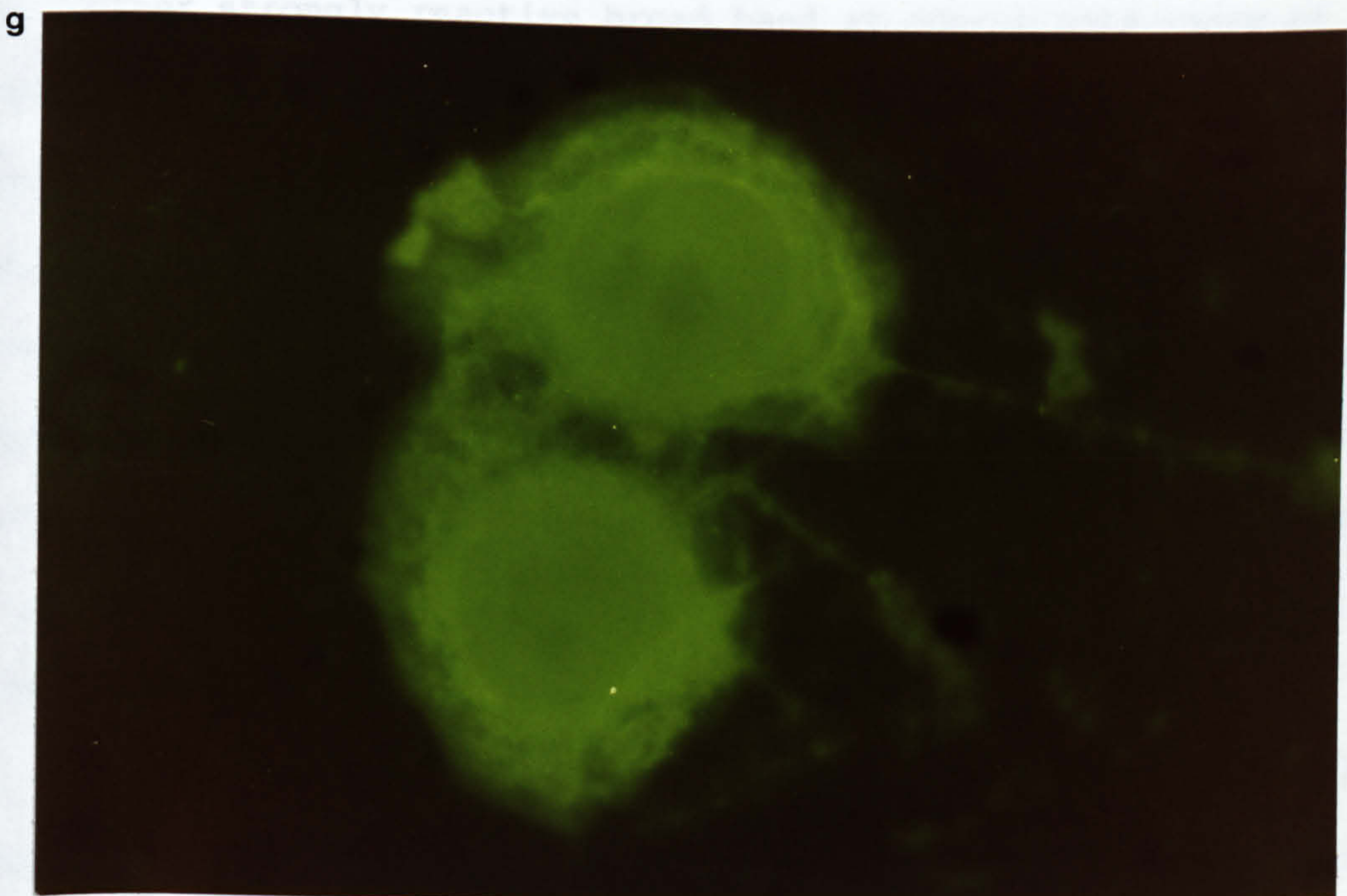
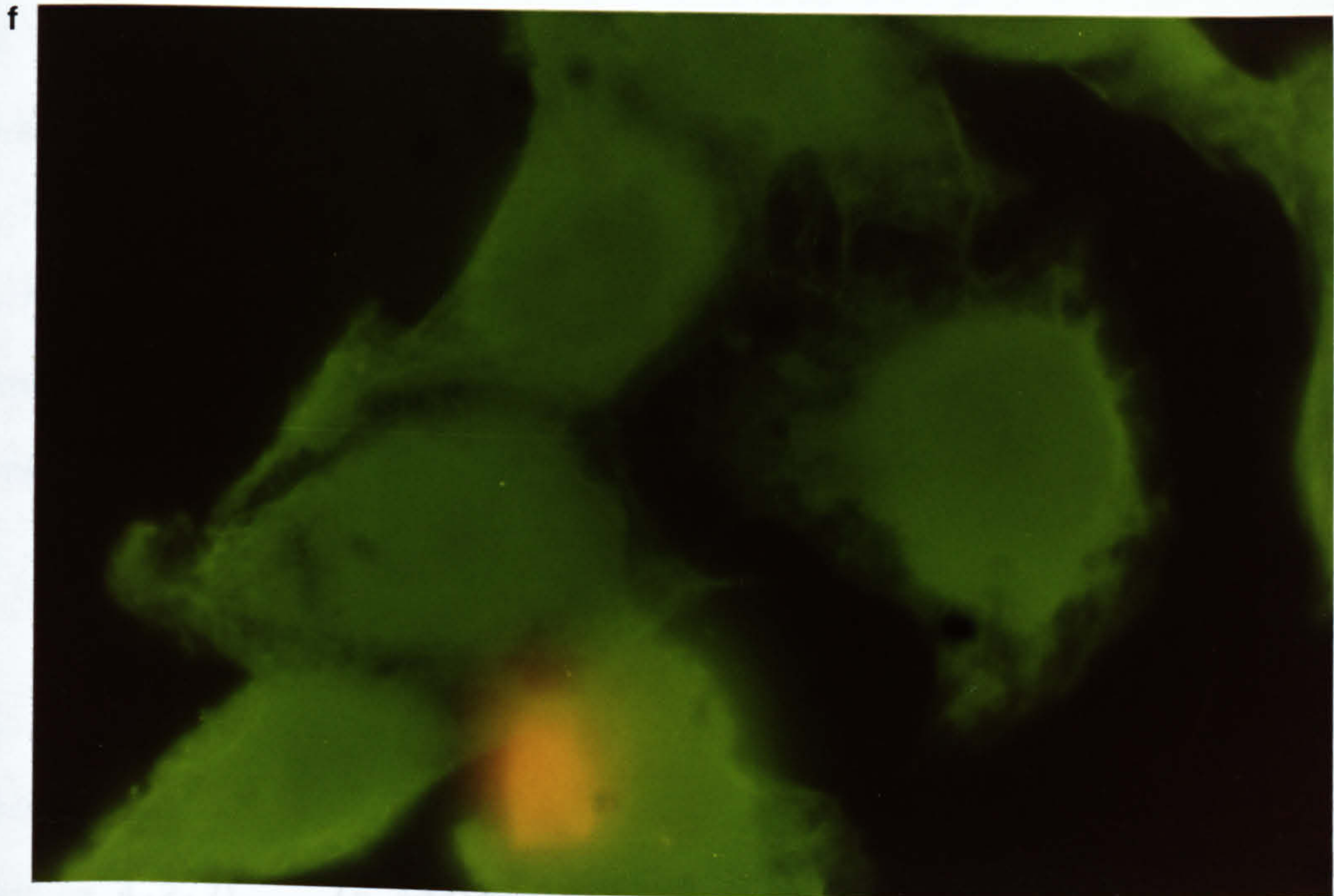
d



e









described in section 3.7.3 and the fluorescent staining is shown in Figure 4.2.6. No distinct filamentous structures were identified although predominant cytoplasmic staining was noted. The intermediate filaments of HeLa cells are largely of the prekeratin and vimentin type and these are both of molecular weights higher than 52,000. Since no strong immunoreactivity of R2.1 antiserum with proteins higher than Mr 52,000 in the chromatin and dehistonised chromatin fractions was detected (Figure 4.2.4), it is likely that the antiserum is either not directed primarily against this type of protein, or that the prekeratin and vimentin groups of cytoplasmic location are probably not contaminating the chromatin preparations.

To clarify this finding, a cytokeratin preparation from whole HeLa cells was run on a gel, blotted onto nitrocellulose paper, and allowed to react with R2.1 antiserum, by the immunoperoxidase method (Figure 4.2.7). In addition to the thick band of Mr 42,000-52,000 (lane 2), another strongly reactive broad band at approximate range of molecular weight of 54,000-61,000 was observed. Another group of proteins in the cytokeratin preparation (lane 2) showed a strong immunoreactivity with R2.1 antiserum at Mr 20,500-24,000. These two groups of proteins were not present in the nuclei and 2M salt-extracted chromatin preparations (lane 3 & 4). The cytokeratin as well as the nuclei preparation (lane 2 & 3) both exhibited another immunoreactivity at approximate Mr of 15,000-16,000, which was absent in the 2M salt-extracted chromatin (lane 4).

Lane (3) in Figure 4.2.7 represents nuclei prepared by the Chauveau method and allowed to react with R2.1 (1/250) antiserum in the immunoblot. A reaction similar to that of the dehistonised chromatin (lane 4) was observed while there was no immunoreactivity with proteins corresponding to molecular weight ranges of 54,000-61,000 and 20,500-24,000. This suggests that there were no reactive proteins in



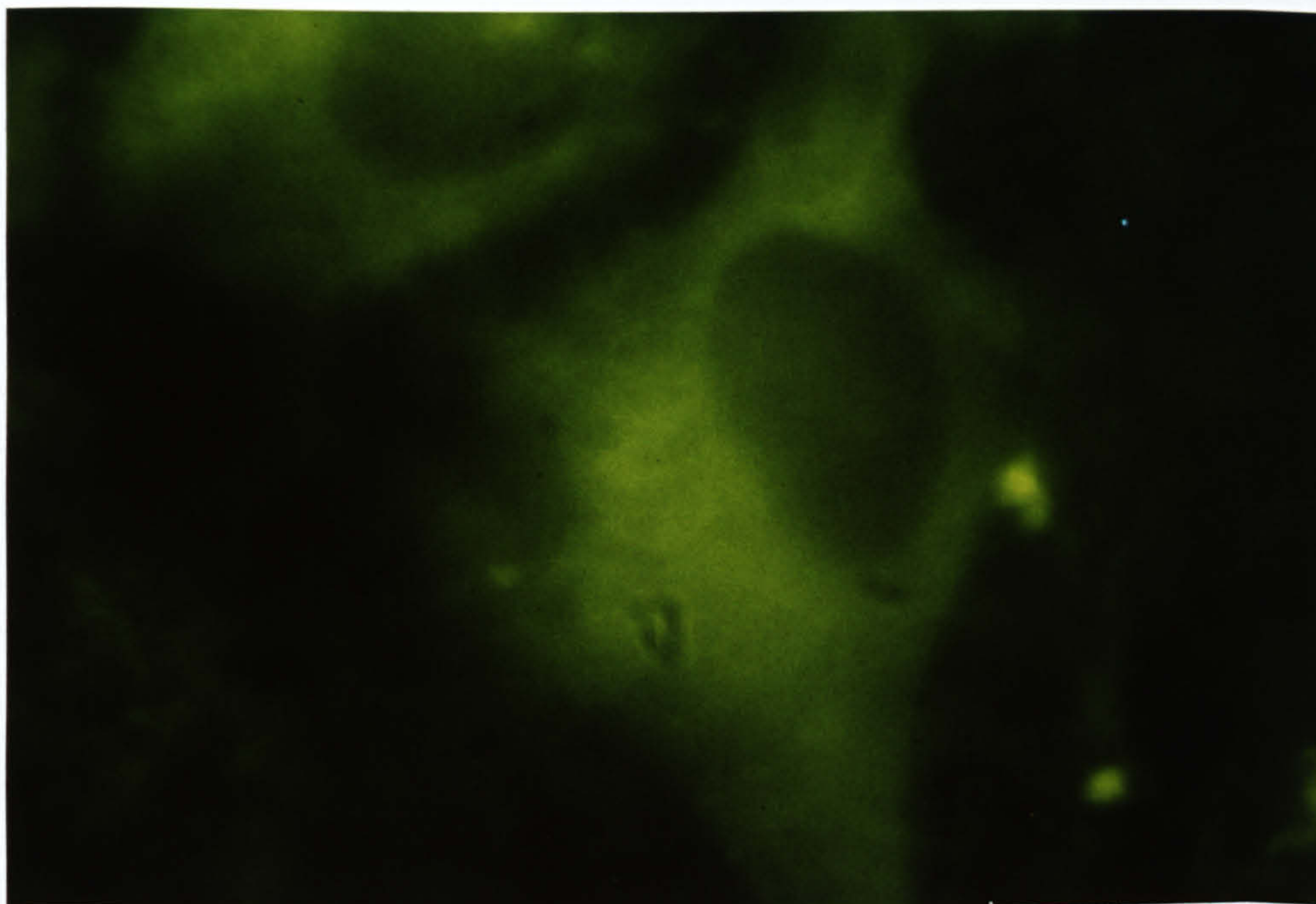
Figure 4.2.6

FITC-staining of HeLa Keratin Structures Reacted  
with R2.1

- (a) An indirect immunofluorescent staining of HeLa cells prepared on coverslips as described in section 3.7.3 preserving the cytokeratins then allowed to react with R2.1 antiserum (1/150). (X2000)
- (b) Phase contrast of cells in a (X2000)



a



b

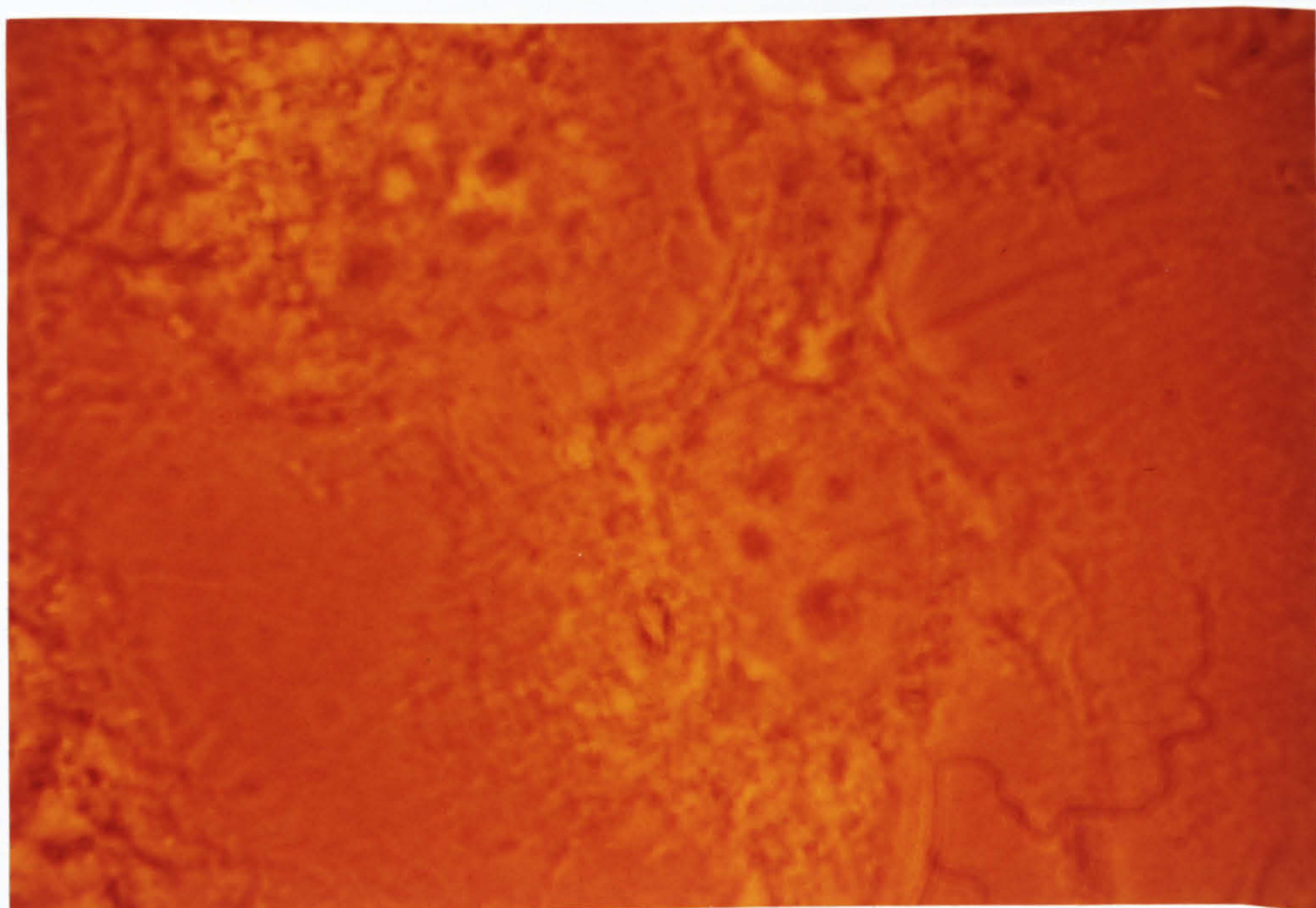




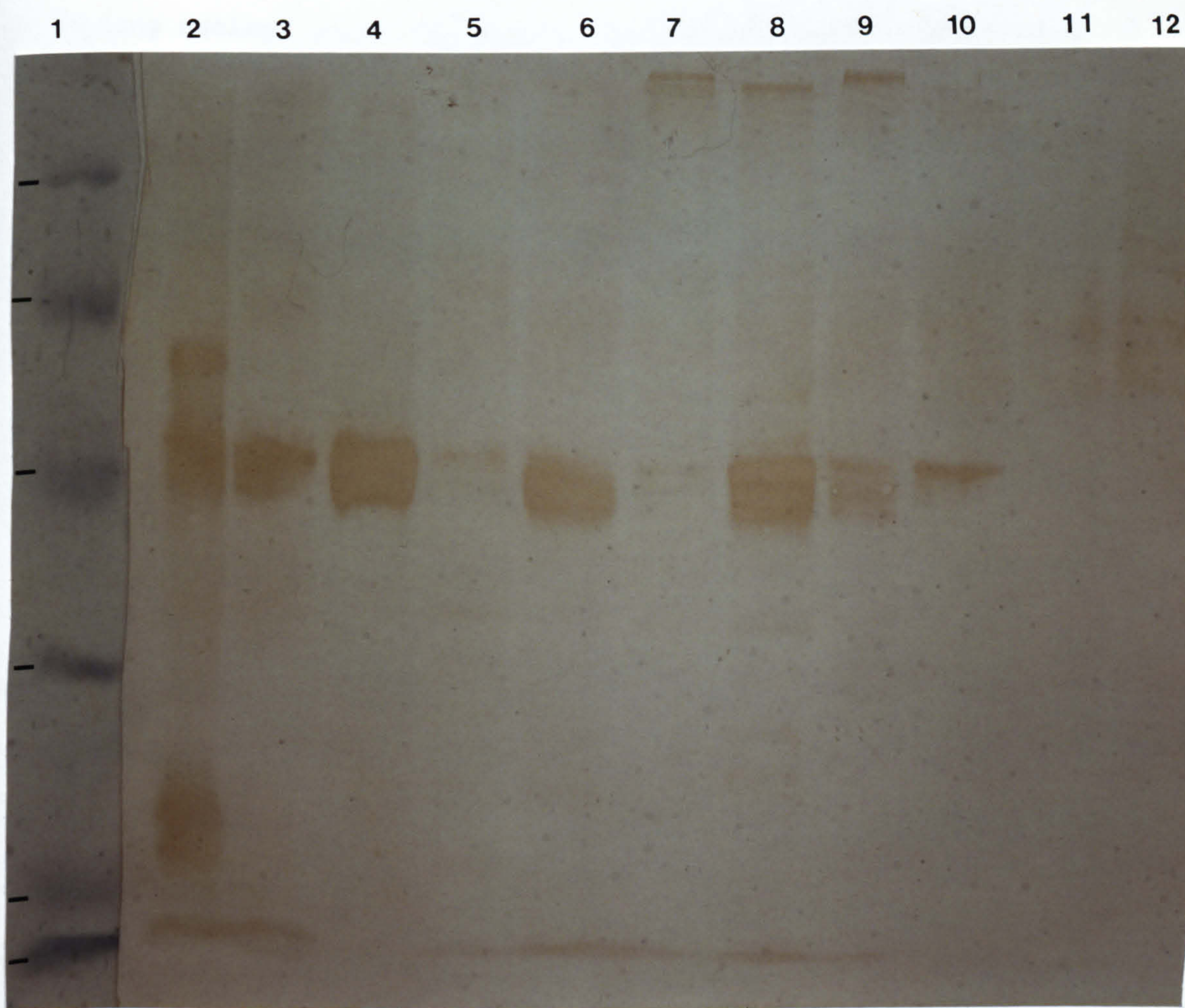
Figure 4.2.7

Immunoblot of R2.1 Reacted with HeLa Subfractions  
Compared with Human and Non-human Dehistonised  
Chromatins

A protein immunoblot (section 3.5.4.1) of R2.1 antiserum reacted with 2M salt-extracted chromatin preparations (lanes 4-12) from different human and non-human cell lines, as well as with HeLa keratin preparations (lane 2) (section 3.8.6) and HeLa nuclei (lane 3) (section 3.1.1.1). Lane (1) is a blot of protein marker stained with amido-black. The protein preparations were run on a 10% Laemmli gel plus 0.5M urea.

- Lanes 1 : protein marker (from top: phosphorylase b 94,000;  
bovine serum albumin 67,000; ovalbumin 43,000;  
carbonic anhydrase 30,000; soybean trypsin  
inhibitor 20,100;  $\alpha$ -lactalbumin 14,400).
- 2 : HeLa keratin preparation
- 3 : HeLa nuclei preparation
- |                 |            |
|-----------------|------------|
| 4 : HeLa        | 5 : K562   |
| 6 : FL          | 7 : KB     |
| 8 : Chang liver | 9 : HT1080 |
| 10 : BSC-1      | 11 : CHO   |
| 12 : BHK-21     |            |







these two ranges of molecular weights, present in the preparation of nuclei.

Whether the cytoplasmic immunofluorescent staining seen in Figures 4.2.5 and 4.2.6 is due to naturally occurring anti-intermediate filament antibodies in the rabbit antiserum or to contaminants in immunising nuclear proteins, remains to be established. However, it is possible that some of these protein antigens are nuclear but because they are synthesised in the cytoplasm, and therefore have been localised there by immunofluorescence. Furthermore, it is also possible that one (or more) of the proteins in the range of Mr 42,000-52,000 is genuinely nuclear but masked by the overlapping of other reactive proteins (of cytoplasmic localisation) which are of similar or approximately similar molecular weights.

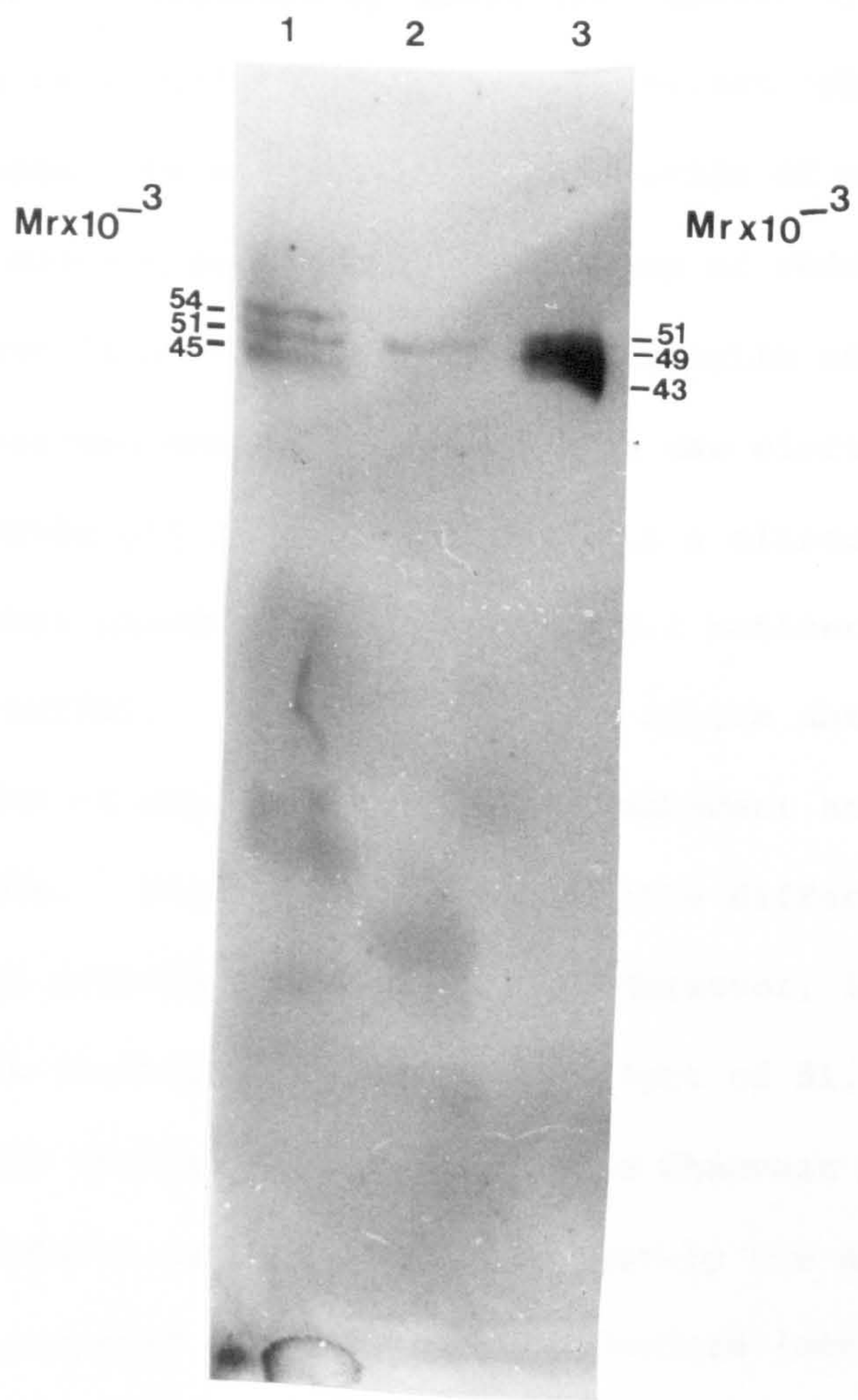
An efficient method of resolving the immunoreactive bands at 42,000-52,000 is by the use of highly purified radiolabelled antibody as shown in Figure 4.2.8. Three highly resolved and sharp bands of molecular weights corresponding to approximately 54,000, 51,000 and 45,000 were clearly observed on the autoradiogram, in the lane which represents the keratin preparation (lane 1). Interestingly, only one single and sharp band at Mr 51,000 could be seen in the nuclear preparation obtained by the method of Chauveau method (lane 2), while in the lane representing the 2M salt-extracted chromatin (lane 3), three bands of approximate molecular weights of 52,000, 49,000 and 43,000 were observed. This may suggest that one protein (51,000) at least could be assigned to the nuclear fraction. The other two proteins in the dehistonised fraction (Mr 49,000 & 43,000) may be present in too small quantities in nuclear preparations, to be detected on the immunoblot. The relative amount of these two proteins may be enriched in the dehistonised chromatin preparation. The molecular weights of these



Figure 4.2.8

Autoradiography of R2.1 Reacted with HeLa  
Subfractions

An autoradiogram of a protein immunoblot of R2.1 antiserum (1/300) described in section 3.5.4.2 reacted with HeLa keratin preparation (lane 1), HeLa nuclei prepared by Chauveau method (lane 2) and 2M salt-extracted chromatin (lane 3). The second antibody (affinity purified anti-rabbit IgG) was labelled by  $^{125}\text{I}$  as described in methods section.





bands were estimated by running a marker protein standard, simultaneously on the same gel. The blot corresponding to the protein marker was stained with amido-black, then aligned with the negative of the autoradiogram to indicate the approximate molecular weight of the reactive proteins.

It is suggested by Lewis and Laemmli (1982) that isolation of the nuclei in a buffer containing polyamines (spermine and spermidine) and digitonin with subsequent sedimentation of nuclear pellet through sucrose gradients results in elimination or reduction of contamination by intermediate filaments. The total chromatin obtained from this method and the Chauveau method of preparation was electrophoresed on a long polyacrylamide gel and transferred onto a nitrocellulose paper. The blot was then allowed to react with R2.1 antiserum diluted 400 times with TN-GS-BSA buffer. The use of a more dilute antiserum should minimise the reaction of non-specific or less abundant antibodies thus reducing interference. Figure 4.2.9 shows little difference between the two preparative methods (lanes 5 & 6). Moreover, it can be seen that only one band is visible at a molecular weight of 51,000 in lane (6) which contains the chromatin obtained by the Chauveau method, while lane (5) showed a broader reaction at approximately the same molecular weight. Chromatin prepared by the routine procedure (section 3.1.2) demonstrated an immunoreactivity at approximate molecular weight of 50,000-51,000 (lane 3). Again, the 2M salt-extracted chromatin (lane 2) showed a strong, broad and unresolved band at a range of Mr 42,000-52,000 which may be due to the enrichment of the other reactive proteins in this kind of preparation. It is worth noting that the cytoplasmic fraction exhibited no detectable immunoreactivity with R2.1 (1/400) antiserum (lane 4).

The results shown in Figure 4.2.8 (lane 2) in conjunction with those presented in Figure 4.2.9 (lanes 3 & 6) confirm that one major nuclear protein is immunoreactive in these subcellular fractions. It is

Figure 4.2.9

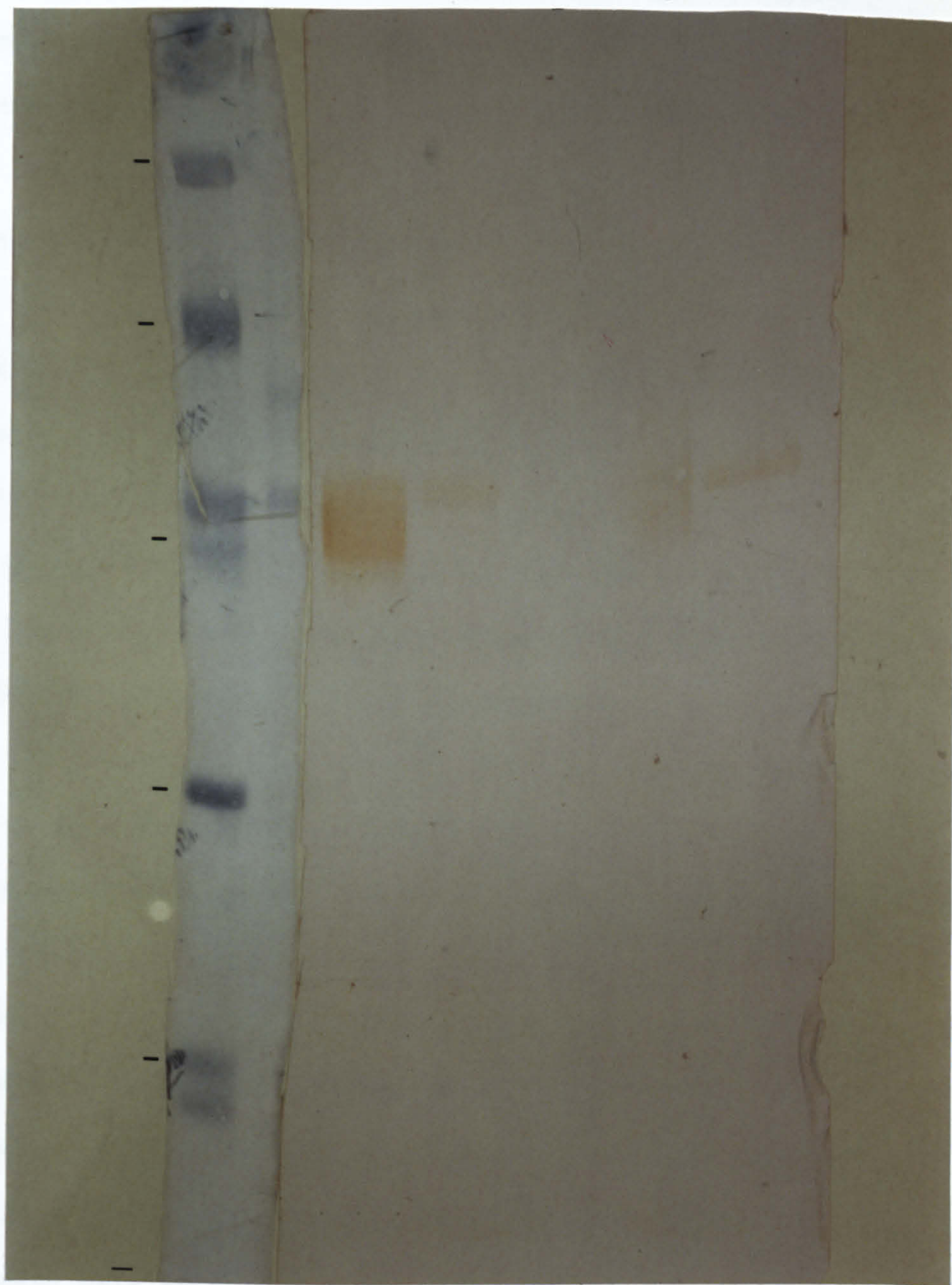
Immunoblot of R2.1 Reacted with HeLa Subfractions

Protein immunoblot of R2.1 antiserum reacted with  
different subcellular preparations from HeLa cells.

- Lane 1 : Amido-black-stained protein marker
- 2 : 2M salt-extracted chromatin
- 3 : Chromatin preparation
- 4 : Cytoplasmic fraction
- 5 : Nuclei prepared by the polyamine method (section  
3.1.1.2)
- 6 : Nuclei prepared by the Chauveau method



1 2 3 4 5 6





also possible to suggest that proteins of approximate molecular weights of 42,000-43,000 and 49,000 usually present in the dehistonised chromatin preparation may be minor components in the total chromatin fraction and become enriched and detectable in the 2M salt-extracted chromatin. However, the possibility that the 43,000 and 49,000 bands are genuinely cytoplasmic proteins, perhaps of the cytokeratin type contaminating the chromatin preparations, can not be ruled out.

Mitotic HeLa cells fixed on coverslips reacted with R2.1 antiserum. Although the intensity of FITC staining in these cells is not as bright as in interphase (Figure 4.2.5), it is clear that all the cells have shown fluorescent immunoreaction (Figure 4.2.10a, c & e). Examination of the swollen mitotic cells revealed unidentified speckled-structures scattered throughout the cell (Figure 4.2.10g). Although the mitotic cells were cytocentrifuged according to the method of Stenman et al. (1975), before reaction with the antibody, in an attempt to spread and detect the chromosomes, the fluorescent staining has not revealed clear metaphase chromosomal structures (Figure 4.2.10g). However, this does not rule out the presence of chromosomal decoration because the antiserum (R2.1) is polyclonal and may have reacted with other components which hinder the clear identification of chromosomal staining.

Figure 4.2.7 shows that other human dehistonised chromatins give different pattern of immunoreactivity with R2.1 antiserum, though there are some similarities. It is interesting to note that the K562 cell line which is a myelogenous leukemia line, despite exhibiting a high reactivity with R2.1 in both C'F and ELISA techniques, shows only one major band at Mr 49,000 (lane 5). The FL cell line which is of an epithelial origin exhibited a broad and unresolved reactive band at Mr 41,000-49,000 (lane 6). KB chromatin (lane 7) reacted less vigorously showing two faint bands corresponding to molecular weights of 45,000 and



Figure 4.2.10

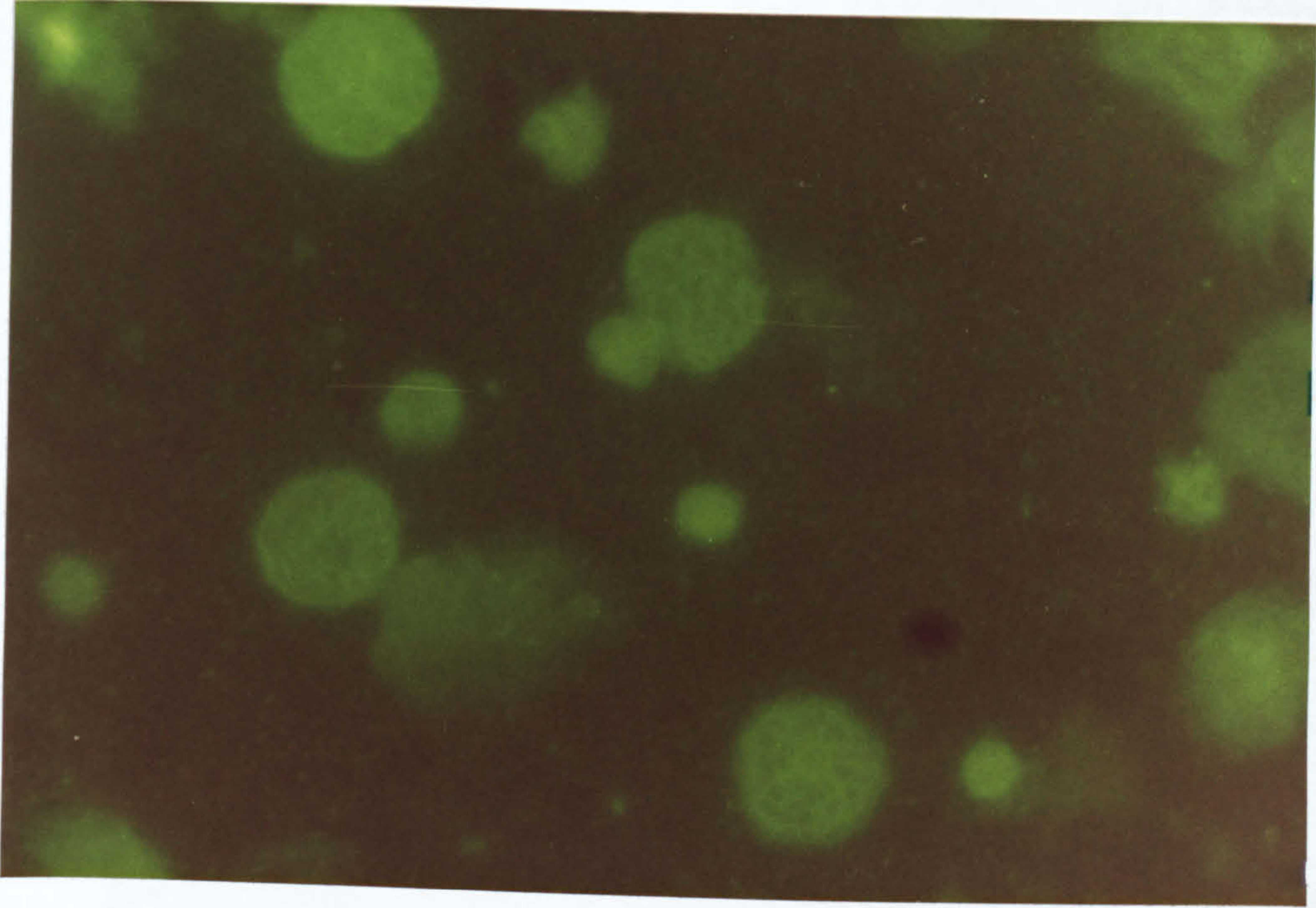
FITC-stained Mitotic HeLa Cells Reacted with R2.1

An indirect immunofluorescent staining of HeLa cells swollen in hypotonic solution and spread and fixed on slides by cytocentrifugation then allowed to react with R2.1 (1/100) antiserum.

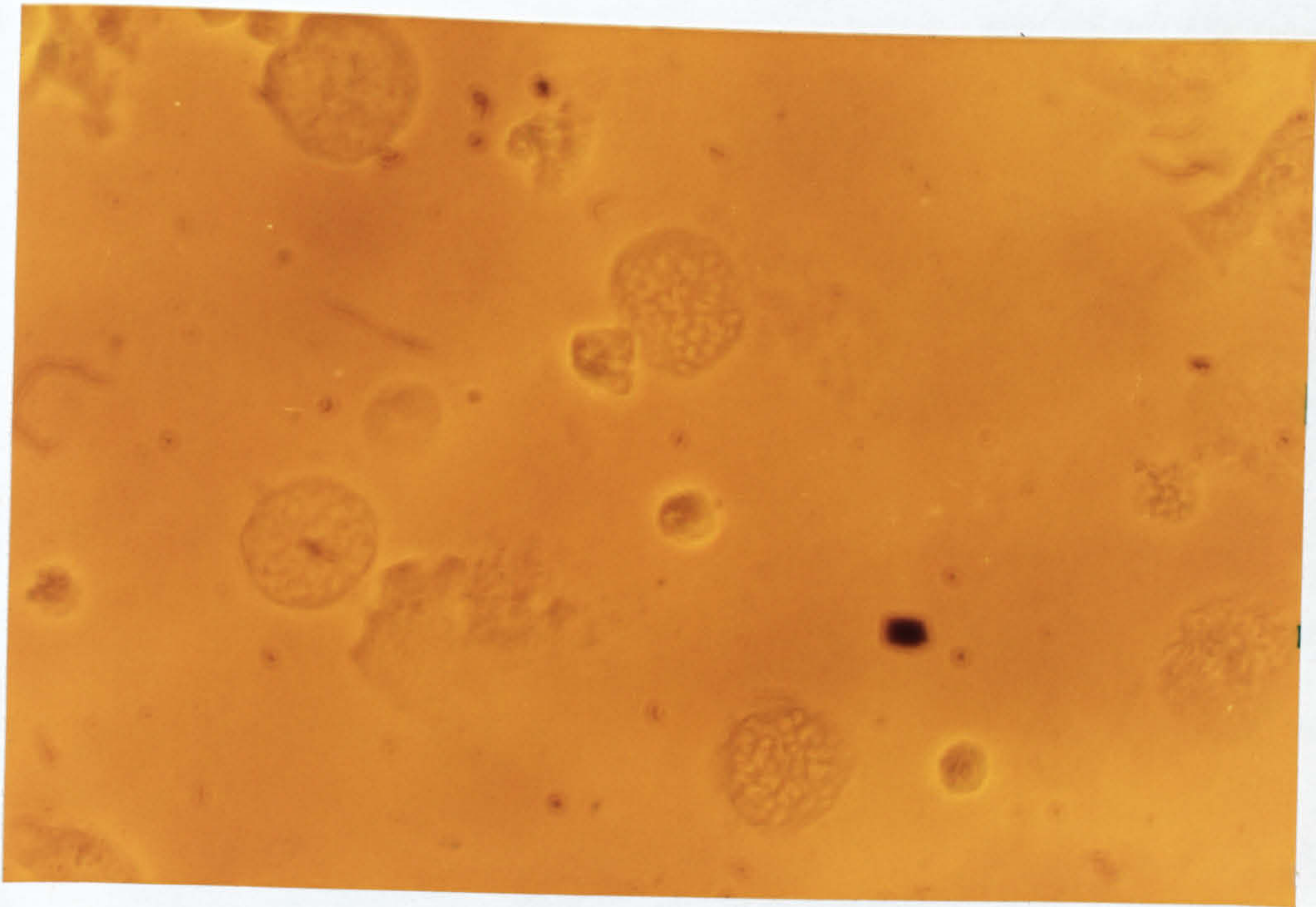
- (a,c & e) : FITC staining (X500)
- (b,d & f) : Phase contrast of cells in a, c and e
- (g) : FITC staining (X2000)



a

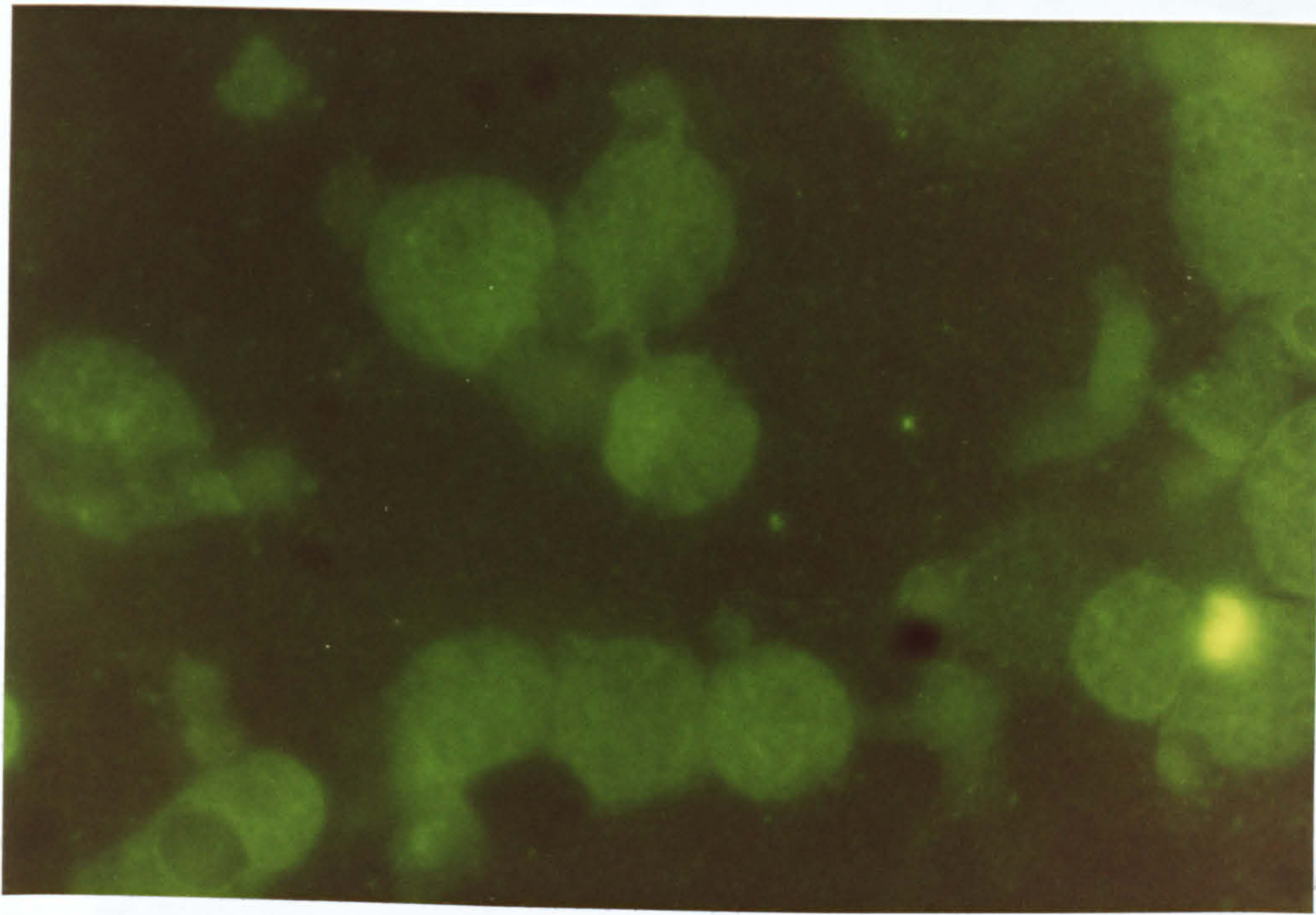


b

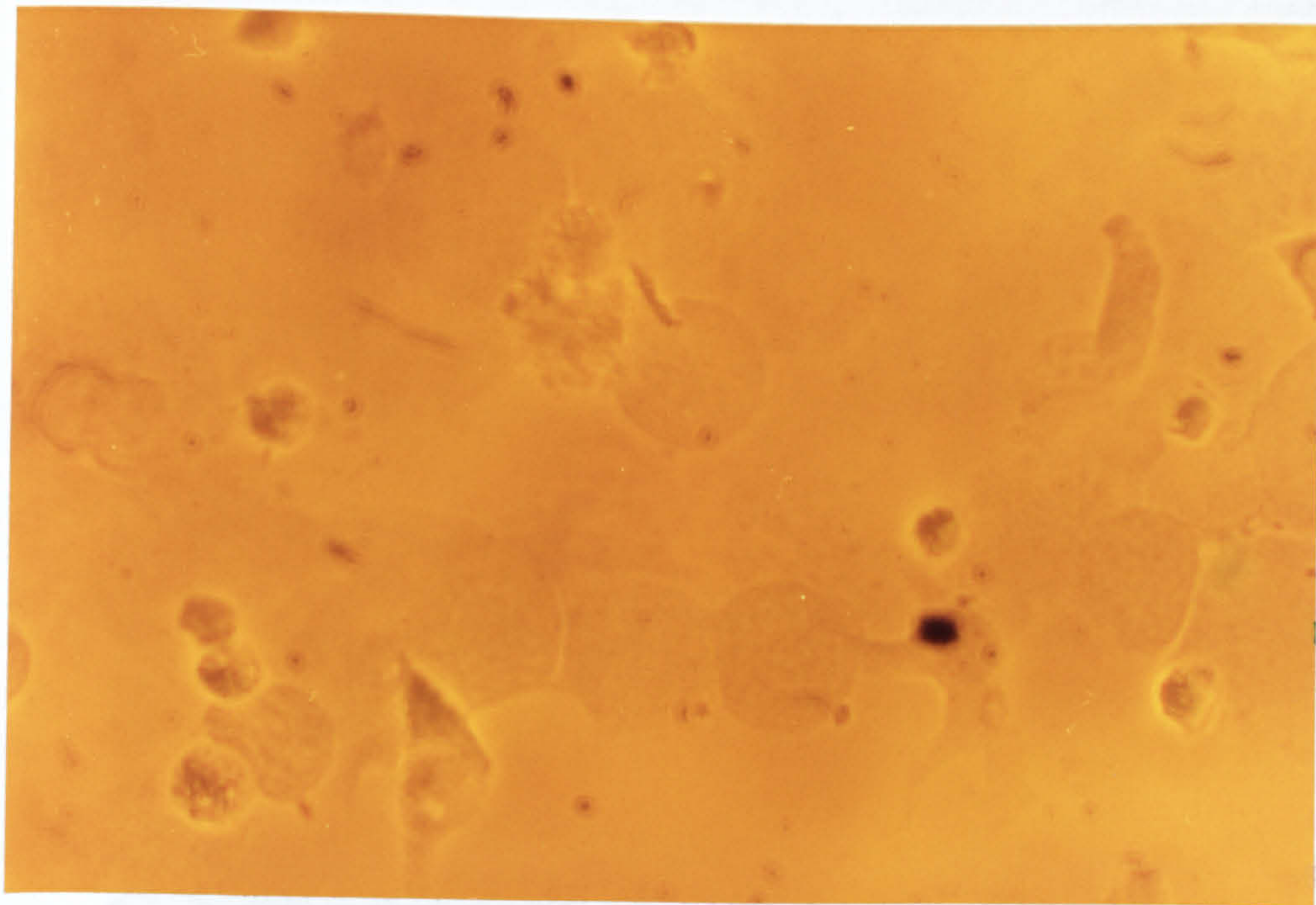




c

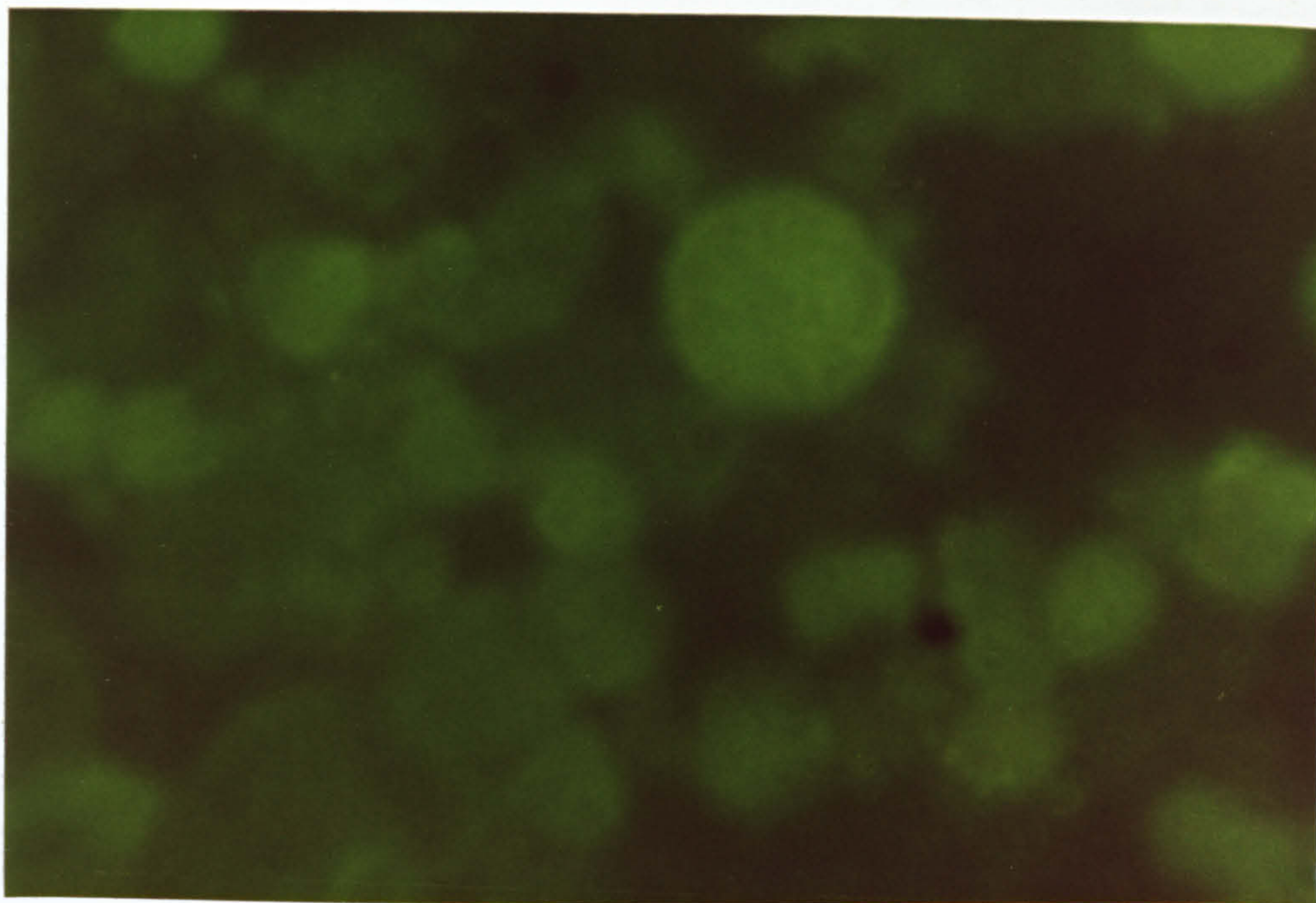


d

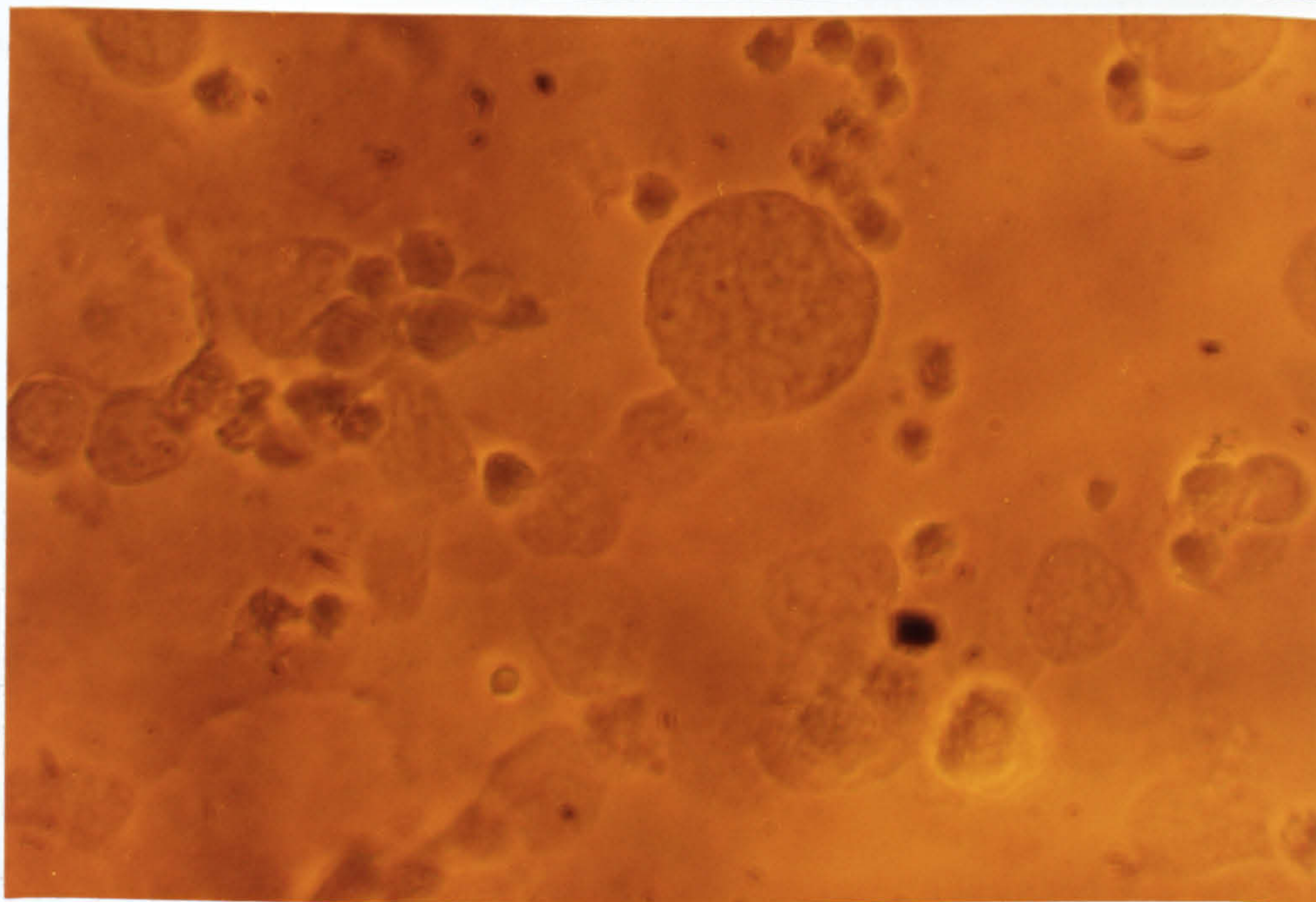




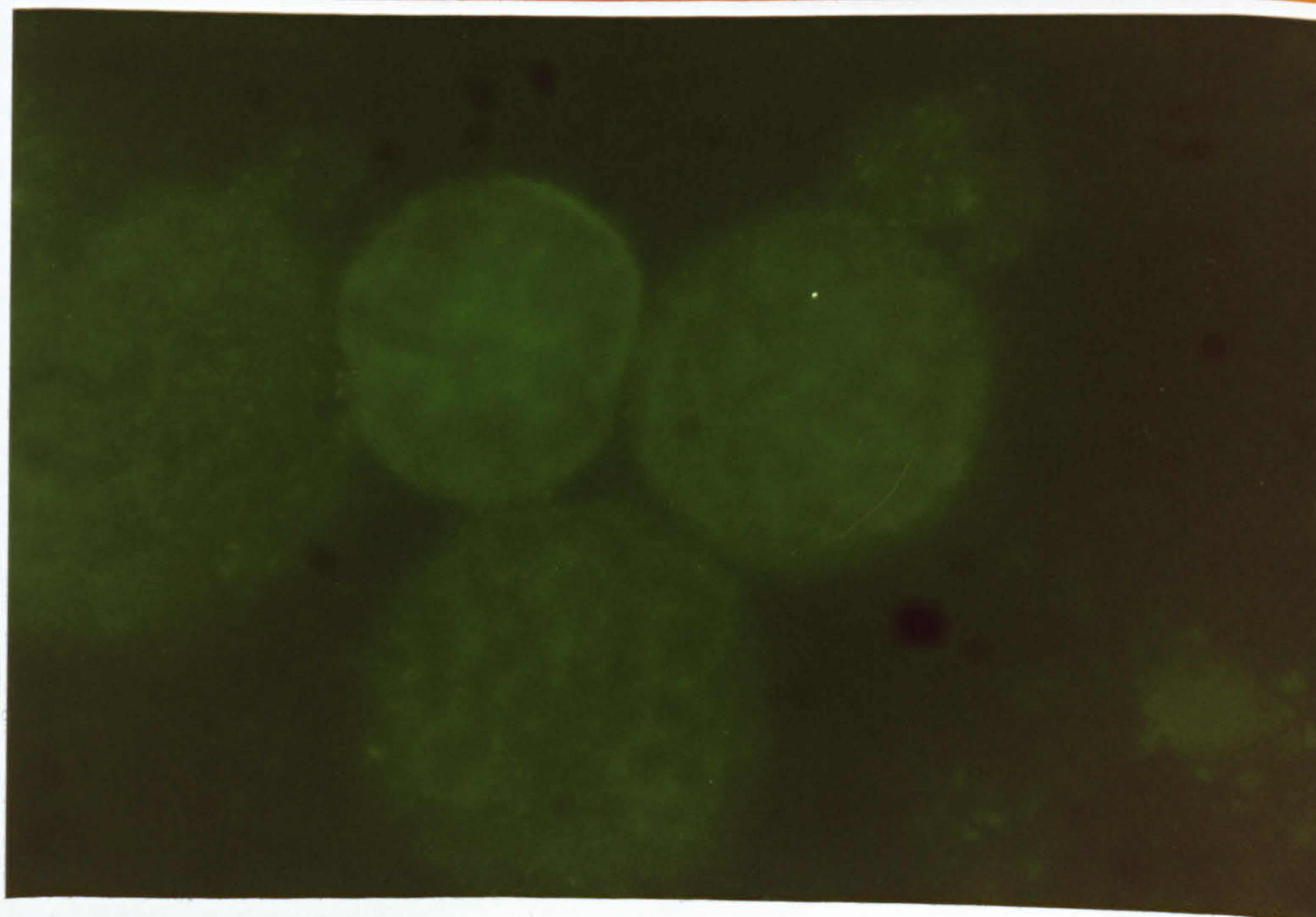
e



f



g





48,000. On the other hand, Chang liver, which showed very little activity in C'F and ELISA, its dehistonised chromatin demonstrated a strong immunoreaction with R2.1 antiserum at Mr 42,000, 45,000 and 49,000 although some overlapping was also noted at the same range of molecular weight. Weak immunoreaction at Mr 51,000 and 59,000 was also observed in the immunoblot (lane 8). The fibrosarcoma cell line, HT1080, showed clear reactive protein bands at Mr 42,000, 44,000 and 49,000 (lane 9).

Of the non-human chromatin, only the green African monkey kidney cell line (BSC-1) showed a clear immunoreaction at 49,000 (lane 10), while both hamster cell lines (CHO & BHK-21) exhibited a weaker reaction corresponding to proteins at molecular weights of 59,000 and 64,000 (lanes 11 & 12).

## Discussion

When the isolated nuclei of HeLa cells were extracted with 2M salt-buffer, a group of proteins believed to have high affinity to DNA could be sedimented through 1M sucrose bed. This 2M salt-extracted chromatin was strongly antigenic. and the resulting polyclonal antiserum (R2.1) was used to determine and characterise the immunologically reactive groups in HeLa chromatin as well as in other human and non-human cell lines, using different techniques (C'F, ELISA, immunoblotting and immunofluorescence). The amount of protein remaining after 2M salt-extraction is greater than the residual protein left behind in 2M salt-5M urea-extraction. Adding to this is the irreversible denaturation of some nuclear components (such as some enzymes) which could be avoided or considered less likely by excluding the urea from the extraction buffer.

Both C'F assay and ELISA (Figures 4.2.1 & 4.2.2) demonstrated very small immunoreactivity between R2.1 and non-human chromatin. On the other hand, different reactivity patterns with respect to human chromatin was observed. Human epithelial cells (Chang liver, KB and H.Ep-2) showed between 20-30% of the reactivity of HeLa chromatin at an antiserum dilution of over 1/200. Exceptionally higher reactivity was exhibited by human blood cell line (K562) in C'F assay (more than 80% C'F) as well as ELISA (approximately 40% of HeLa chromatin reactivity).

Unlike the immunoreactivity of the antigen-complex seen with R3.1 antiserum and those reported by Campbell et al. (1979) which were raised against 2M salt-5M urea-extracted chromatin, only a third of the immunoreaction of R2.1 with HeLa chromatin was lost <sup>after</sup> extensive micrococcal nuclease digestion (Figure 4.2.3). The lost immunoreactivity was again almost restored on reconstituting the digested chromatin with HeLa DNA, while the DNA alone failed to exhibit any significant reaction with R2.1 antiserum.



The need for DNA association (for part of the antigen) was once again demonstrated when a group of immunologically reactive proteins showed a faint reaction after treating the immunoblot with HeLa DNA prior to its reactivity with R2.1 antiserum. These reactive groups were seen in both chromatin and dehistonised chromatin fractions (Figure 4.2.4). The first group which was not clearly resolved on the blot occupy a range of molecular weights of 52,000-64,000. The other DNA-dependent reactive groups could be seen (arrows) at higher molecular weights (approximately Mr 80,000 & 100,000). A third and again unresolved group which showed clearly strong reaction with R2.1 antiserum after treating the blot with HeLa DNA was also observed at a range of Mr 40,000-42,000, overlapping the other group (42,000-52,000) which did not require DNA-association for its immunoreaction.

There is a great difference in the intensity of reaction between the HeLa interphase and metaphase cells as viewed by FITC staining (Figure 4.2.5 & 4.2.10). This difference could be attributed to the absence (or decrease) of some components of the reactive antigen(s) during mitosis. The pattern seen in mitotic cells did not show any distinct FITC-stained chromosomal structures. This may be due to the overlapping of chromosomal and non-chromosomal reactions.

It is obviously relevant to determine whether all the reactive groups seen on the immunoblot are nuclear, or cytoplasmic. The risk of cytoplasmic contamination stems mainly from a group of proteins (collectively called cytokeratins and specifically termed intermediate filaments - I.F.) which resist solubilisation by non-ionic detergents (such as triton X-100) and by low or high salt-extraction (reviewed recently by Moll et al. 1982 & Lazarides 1982).

Although Franke et al. (1979) have reported the presence of only four major intermediate filament protein bands in HeLa cells of Mr 54,000, 52,000, 48,000 and 46,000, recent reports have demonstrated

another two immunologically reactive protein groups at Mr 43,000 and 50,000 (Bravo et al, 1983) and at 58,000 and 65,000-67,000 (Tseng et al. 1982).

Figure 4.2.7 (lane 2) shows a cytokeratin preparation from HeLa cells reacting with four groups of proteins on the immunoblot. Comparing these immunologically reactive groups with that of the dehistonised chromatin (lane 4), it is possible to detect that only one group (42,000-52,000) is common to both preparations, while the other three groups, (54,000-61,000, 20,500-24,000 & 15,000-16,000) are confined to the keratin preparation. The chromatin preparation (Figure 4.2.4) exhibited a reaction pattern exactly similar to the dehistonised fraction (lanes 6 & 7). Unlike the keratin preparation (Figure 4.2.7, lane 2), no other reactive group (other than the 42,000-52,000 group) could be seen in either of these two preparations (chromatin and dehistonised chromatin; Figure 4.2.4, lanes 6 & 7) nor in the nuclear fraction (except the Mr 15,000-16,000 group) seen in Figure 4.2.7, lane (3).

It is important to emphasise that HeLa intermediate filaments have been shown to be distinct (Franke et al. 1979; Moll et al. 1982). It is also noteworthy that antisera and monoclonal antibodies produced by immunising the animals with dehistonised chromatin fraction have also exhibited specific reactivity with intermediate filament class of proteins (Schmidt et al. 1981; Turner, 1981).

The major cytokeratin proteins in HeLa cells (vimentin and phosphovimentin) are of Mr 54,000-55,000, and another two cytokeratins of Mr 52,000 and 58,000 have been identified (Bravo & Celis, 1982). Consequently, some of the reactive proteins (which reacted with R2.1) in this region of the keratin preparation (Figures 4.2.7, lane 2 & 4.2.8 lane 1) may be assigned to cytoplasmic reactive groups including intermediate filaments. This could also be correlated with some of the cytoplasmic reaction seen by immunofluorescent staining (Figure 4,2,5 &



4.2.6). Since there are no distinctly defined filaments or meshwork arrays or fibrils, it is difficult to speculate whether these fibrous structures are of the intermediate filament type or of actin (Mr 43,000). Hindering this identification is the possibility of a mixture of a number of cytoplasmically reactive groups, present together, which mask the presence of a defined filamentous organisation.

The relative intensity of the reactive group in the dehistonised chromatin fractions, shown in Figure 4.2.7, implies that the HeLa fraction (lane 4) has approximately the same immunoreactivity as that of Chang liver chromatin (lane 8). This is in contrast to the results shown in Figure 4.2.2a where HeLa chromatin demonstrated very high immunoreaction with R2.1 antiserum, in comparison with Chang liver chromatin. The latter demonstrated only 20% of HeLa chromatin immunoreactivity. This could be explained as follows:

- 1) Some of these reactive groups are of intermediate filament (I.F.) contaminants which are common to both cell lines. Their contribution (I.F.) to the immunoreactivity of HeLa chromatin is probably equivalent to the observed 20% exhibited by Chang liver chromatin.
- 2) Much of the specific HeLa antigen immunoreaction is associated with a) DNA-complex formation. Evidence supporting this possibility is that part (at least 30%) of the HeLa antigen requires DNA-association to exert its immunoreaction with R2.1 antiserum (Figure 4.2.3), and b) chromatin components of a complex of components which are more abundant in HeLa chromatin than in Chang liver. Upon 2M salt extraction of both chromatins, enrichment of the nonspecific components has taken place in both dehistonised fractions (HeLa & Chang liver). These components may be common to many epithelial and related cell lines (HeLa, Chang liver, FL and HT1080). This would result in overlapping between the chromatin-associated reactive proteins and the cytoplasmic contaminants in a broad reactive band, and indicate that the dehistonised fraction of

HeLa chromatin exercises a similar reactivity pattern with those of other epithelial cell lines on the blot.

Amongst the reactive group of Mr 42,000-52,000 in HeLa chromatin preparations, at least one band (Mr 51,000) is genuinely a nuclear protein (Figures 4.2.8 lane 2 & 4.2.9 lane 3 & 6). The rest of the reactive proteins in this region are either cytoplasmic contaminants or a result of overlapping between nuclear and non-nuclear bands. The FITC-staining of whole HeLa cells (Figure 4.2.5) indicates that the predominant immunofluorescence is concentrated in the nuclei. This implies that the majority of the reactive antigens are of nuclear localisation. Further support to this idea is the DNA-requirement for part (30%) of the HeLa chromatin reactivity demonstrated in Figures 4.2.3 and 4.2.4, and also by the observation in Figures 4.2.8 and 4.2.9 which shows that the Mr 51,000 protein is nuclear associated. Thus the nuclear antigen visualised by FITC-staining is very probably from two or more sources:-

- 1) Nuclear protein(s) represented mainly by Mr 51,000 which do not require complex formation with DNA for immunoreactivity. The presence of other reactive nuclear protein(s) of this class in the region of 42,000-52,000 is highly possible (Figure 4.2.8 lane 3).
- 2) Nuclear antigens which require the presence of DNA-complex formation for immunoreactivity. These antigens are represented by
  - (a) strongly reactive proteins, Mr 40,000-42,000 (Figure 4.2.4 lane 9).
  - (b) very weakly reactive bands at Mr 80,000 and above 100,000.
  - (c) intermediately reactive protein bands at Mr 52,000-64,000.

The last finding is in agreement with the report of Lebkowski and Laemmli (1982) in which it is emphasised that major DNA-binding proteins, in 2M salt-extracted HeLa chromatin, are of molecular weights of 60,000-70,000 and include bands at Mr 60,000, 68,000 and 70,000 and a minor protein at Mr 64,000.



In summary it can be suggested that R2.1 antiserum, which has been raised in a rabbit against 2M salt-extracted chromatin, reacts with two different groups of proteins in HeLa cells.

1. Nuclear proteins: This group is of two types:

(a) Nuclear proteins whose immunoreactivity necessitates DNA-binding in order to dictate the required antigenic determinants presentation for R2.1 reaction. Some of these proteins, are possibly the type of nuclear laminar proteins (Mr 60,000-70,000) described by Lebkowski and Laemmli (1982).

(b) Nuclear proteins which react with R2.1 antiserum regardless of the presence of DNA-binding.

2. The second group is that of cytoplasmic localisation. This group again could be divided into two subgroups.

(a) Those of cytoplasmic contaminants represented mainly by the cytokeratins or other filamentous-forming proteins.

(b) Those of nuclear origin but reacted in the cytoplasm because they are synthesised there.

It is also interesting to note that Adolph (1980a) has reported that a cluster of the scaffold proteins in HeLa cells is of the approximate molecular weight range of 50,000-55,000. Although this report has been contradicted recently by Lebkowski and Laemmli (1982) who suggested that these proteins represent intermediate filaments contamination, Figures 4.2.4, 4.2.8 and 4.2.9 indicated that a protein of Mr 51,000 is amongst the residual nuclear scaffold components. In addition, DNA-binding proteins (Figure 4.2.4) are also among the residual proteins and could be immunologically localised at a molecular weight range of 52,000-64,000. This is also in agreement with Adolph's previous report (Adolph, 1980a) as well as with the suggestion of

Lebkowski and Laemmli (1982), since the DNA-binding proteins recognised by R2.1 reaction accommodate parts of the two groups of proteins (50,000-55,000 and 60,000-70,000) reported by Adolph (1980a) and Lebkowski & Laemmli (1982) respectively.



#### 4.3 Results and Discussion of Monoclonal HNo-G7

HNo-G7 is a monoclonal antibody resulting from the fusion of spleen cells from a BALB/c mouse immunised with dehistonised HeLa chromatin and X63-Ag8-653 (653) plasmacytoma cells. The fusion schedule is outlined in Figure 4.3.1a. The murine hybridoma cell line was cloned four times by limited dilution at 0.5-1 cell/well and has been established in culture for more than 18 months without loss of secretion. The established clone can be frozen and recovered from liquid nitrogen and when grown to exhaustion secretes  $30\mu\text{g IgM}/10^6$  cells/day as estimated by ELISA with reference to controls of standard mouse IgM. Recloning shows no revertant colonies and the line is now assumed to be stable.

The hybridoma line which produces the cells is shown in Figure 4.3.1b forming a colony.

HNo-G7 was shown to be from an IgM-secreting hybridoma both by ELISA assays utilising class-specific antisera and internal radio-labelling (Fig. 4.3.2).

The reaction of the monoclonal antibody and its unknown antigen was initially investigated with respect to the pH of the ELISA assay since other monoclonal antibodies have been shown to have very precise assay requirements in this respect and these would clearly affect subsequent characterisation (Fraser et al., 1982; Mossman et al., 1980). Figure 4.3.3 shows that the pH does not have a substantial effect on the assay of the monoclonal antibody with immobilised HeLa chromatin at pH7-11. Figure 4.3.4 demonstrates that, although the binding to the plate differs at various pH values, it is at saturation at the usual concentrations of antigen employed ( $10\mu\text{g}/\text{well}$ ). The difference at low antigen concentration could also be attributed to the buffer system used for immobilising the antigen, but this again has no significant effect on the course of this study since carbonate is the routinely used buffer.

Figure 4.3.1a    Fusion Schedule

<u>Step</u>	<u>Day</u>	<u>Operation</u>
1	-28	Injection with 100µg DNA of dehistonised chromatin prepared by 2M salt - 5M urea extraction (see section 3.2.2) in complete Freund's adjuvant, intraperitoneally.
2	-18	Injection with 100µg DNA of dehistonised chromatin as above.
3	-5	Injection of 100µg dehistonised chromatin in saline, intraperitoneally.
4	0	Fusion (see section 3.3.5).
5	14-18	Assay. Positive clones selected subcloned at 0.5-1 cell/well.
6	30-34	Clones assayed. Positives selected and subcloned at 0.5-1 cell/well.
7		Steps 5 & 6 repeated twice.



Figure 4.3.1b    Hybridoma Colony of HNo-G7

HNo-G7 Hybridoma colony visualised under Light Microscope  
(X125)







Figure 4.3.2      Immunoglobulin Secretion of HNo-G7

Immunoprecipitate of secreted immunoglobulin (internally labelled with [<sup>35</sup>S]-methionine) from the P3-X63-Ag8(IgG secreting) myeloma cell line (lane 1) and HNo-G7 Hybridoma (lane 2) analysed on a 10% SDS-PAGE. The procedures of radiolabelling and immunoprecipitation are described in section 3.5.2. The gel mixture is of a ratio of 0.8:30 (bisacrylamide:acrylamide)

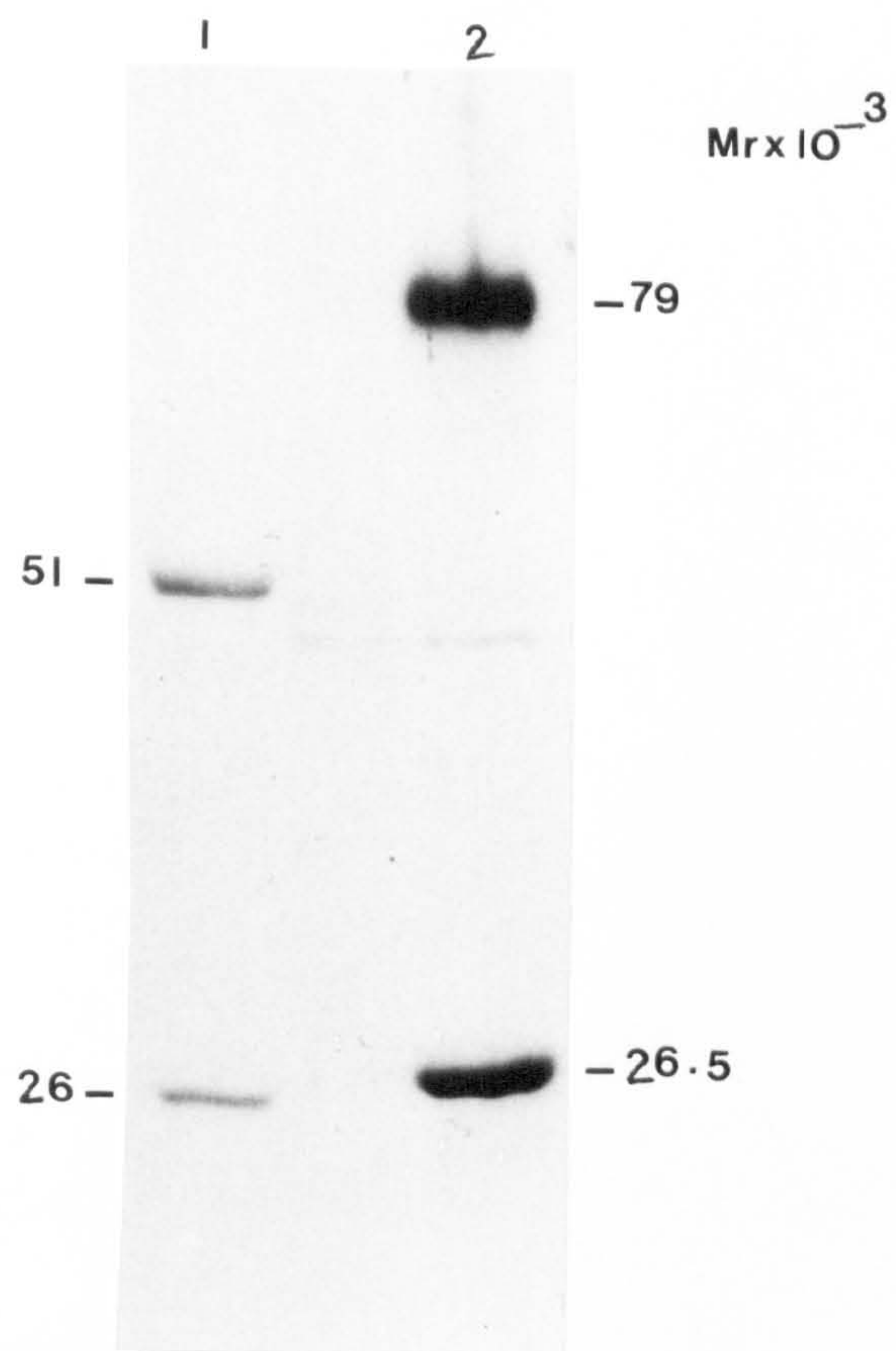




Figure 4.3.3     pH Effect on HNo-G7 Immunoreactivity

The effect of pH on the immunoreactivity of HNo-G7 with HeLa chromatin as tested by ELISA (Procedure is described in section 3.4.4).

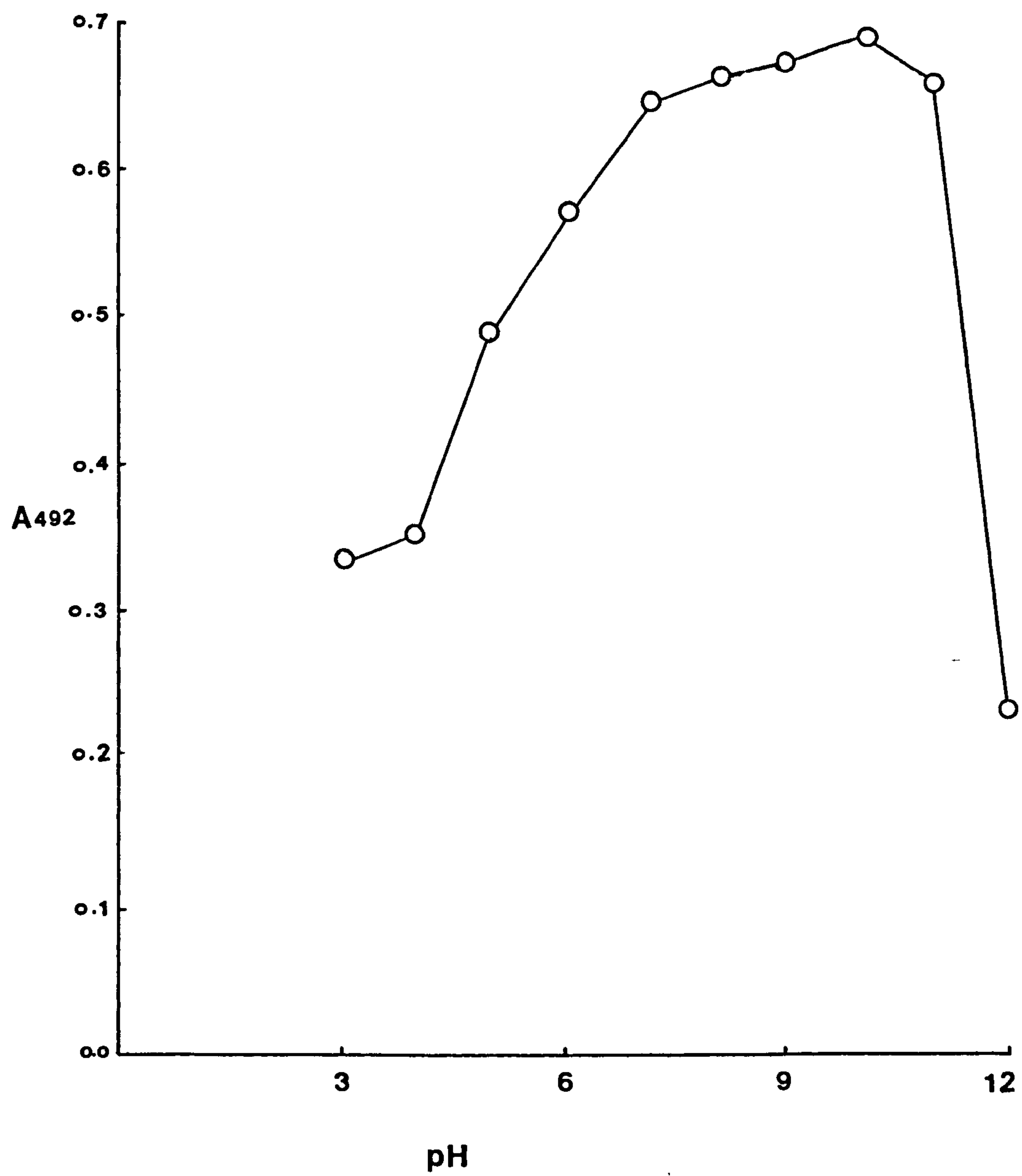
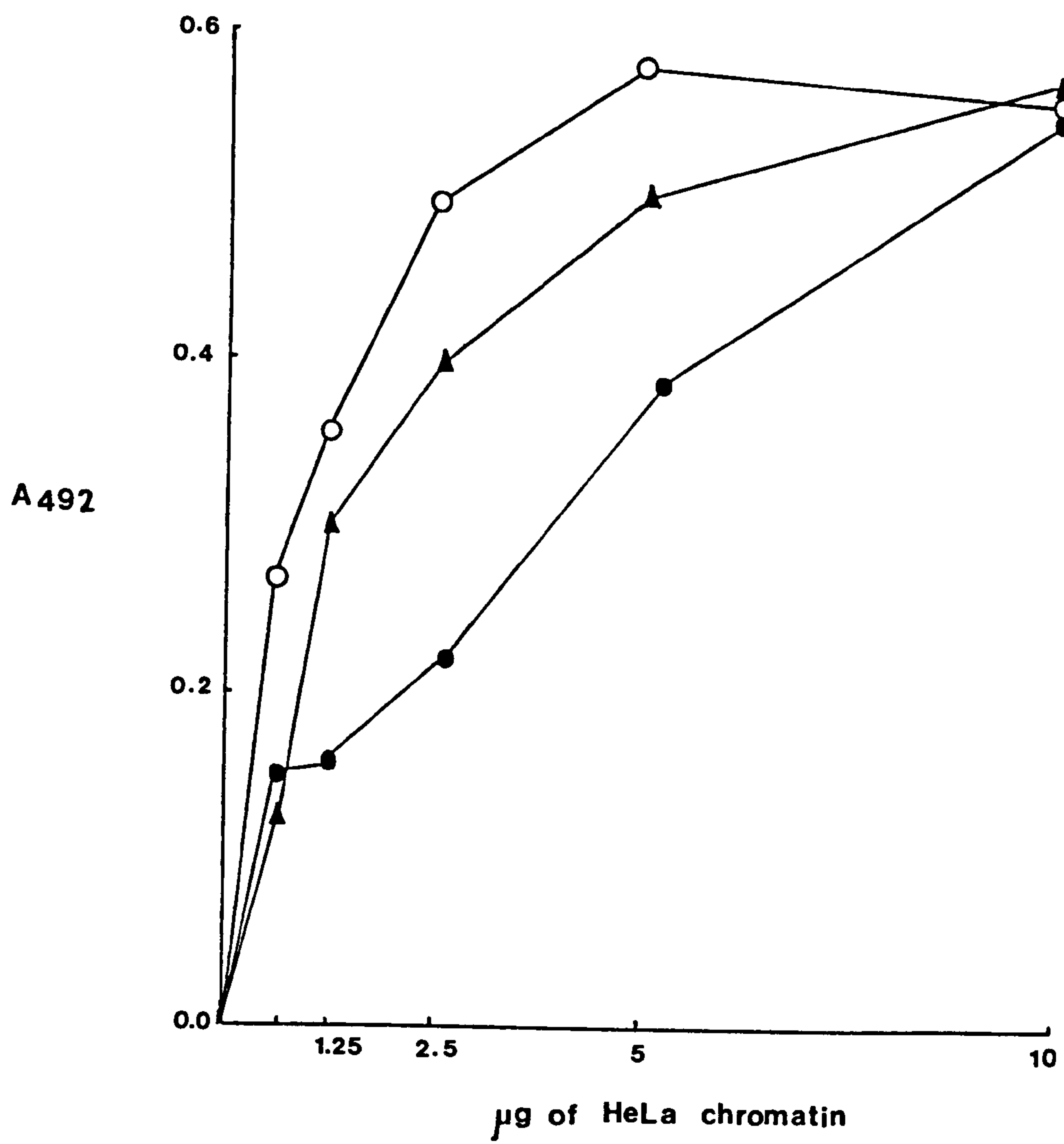




Figure 4.3.4      Effect of Ag- Coating Buffer

ELISA of HNo-G7 antibody reacted with various amounts of HeLa chromatin, suspended and immobilised in the ELISA plate using different coating buffers.

- In carbonate buffer pH9.5  
▲ In PBS pH7.3  
● In TN buffer pH7.4





The "Chequerboard" ELISA of the monoclonal antibody with the antigen (Fig. 4.3.5.a) is performed to give an indication of the dilutions of culture supernatant which may be used for assay of both antibody and antigen. While visual inspection of the plate gives a clear indication of the results, the data are also read by "Titretrek Multiskan Spectrophotometer" to give a more detailed analysis and laboratory record. Figure 4.3.5b shows selective reading from the above "Chequerboard". It is clear that at excess antibody (diluted up to 1/64) there is only small difference (less than 0.2 A492 units) between the reactivity of HNo-G7 with various concentrations of HeLa chromatin above 2.5µg/well. This again indicates that a near saturation of antigen binding is reached when more than 2.5µg/well of chromatin is layered on the ELISA plate.

The specificity of HNo-G7 was then tested by its reaction with other human chromatins in comparison to HeLa chromatin. Figure 4.3.6 shows the reaction in comparison to two other established human epithelial cell lines (H.Ep-2 & Chang liver) and a fibrosarcoma line, HT1080. It is clear that either the same antigen or the same determinant is present in the other epithelial cell lines but that there is no detectable antigenic activity in the fibrosarcoma cell line. It is also clear that the antigenic determinant is not present on purified human DNA.

The quantitative difference between the various types of chromatin could reflect differential binding of the antigen to the ELISA plate. Consequently, a competition assay as described in section 3.4.5 was also employed to examine this phenomenon. HeLa and other human chromatins at different concentrations were incubated at 4°C for 18-24 hours with a fixed amount of HNo-G7 culture supernatant diluted 1/20 to absorb the antibody. The mixture was centrifuged briefly and 50µl of a supernatant

Figure 4.3.5a    A Chequerboard ELISA of HNo-G7 with HeLa Chromatin

ELISA of different dilutions (1-1/64) of HNo-G7 antibody (lanes B→H) with various concentrations (40µg-0.08µg) of HeLa chromatin (lanes 3→12) on an ELISA plate. Wells numbered A3→A12 represent the antigen control of various amounts of HeLa chromatin, while antibody controls of different dilutions are represented by wells numbered 2B→2H. Well number A2 was not treated initially with either antigen or HNo-G7 but at the final stage of the procedure was incubated with anti-mouse-IgG-peroxidase-conjugated antiserum.



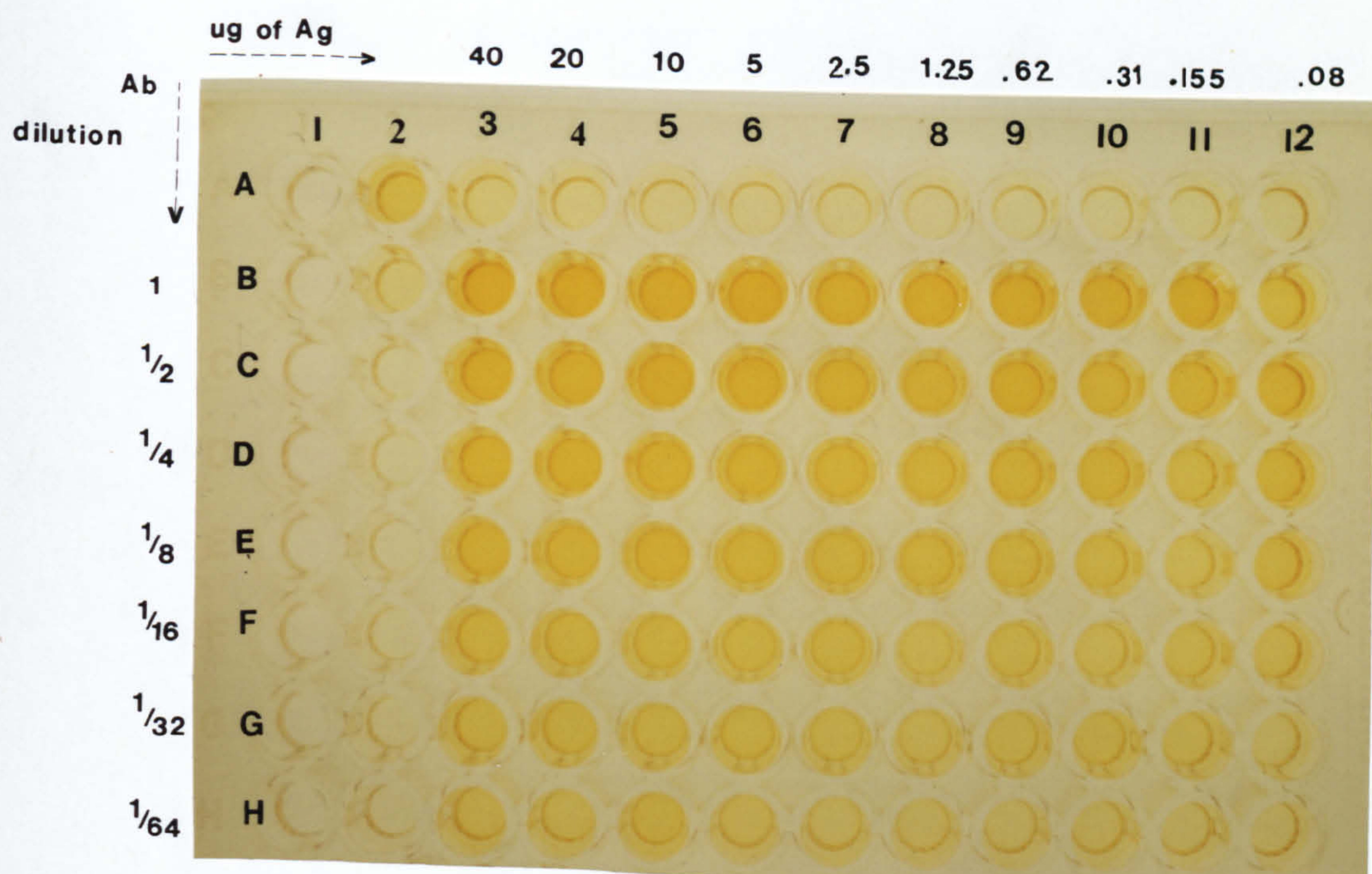




Figure 4.3.5.b ELISA of HNo-G7 with HeLa Chromatin

ELISA of different dilutions of HNo-G7 antibody with various concentrations of HeLa chromatin selected from the "chequerboard" test in Figure 4.3.5a. Antibody and antigen controls were subtracted.

□ HNo-G7 antibody diluted 1/16

○ HNo-G7 diluted 1/32

▲ HNo-G7 diluted 1/64



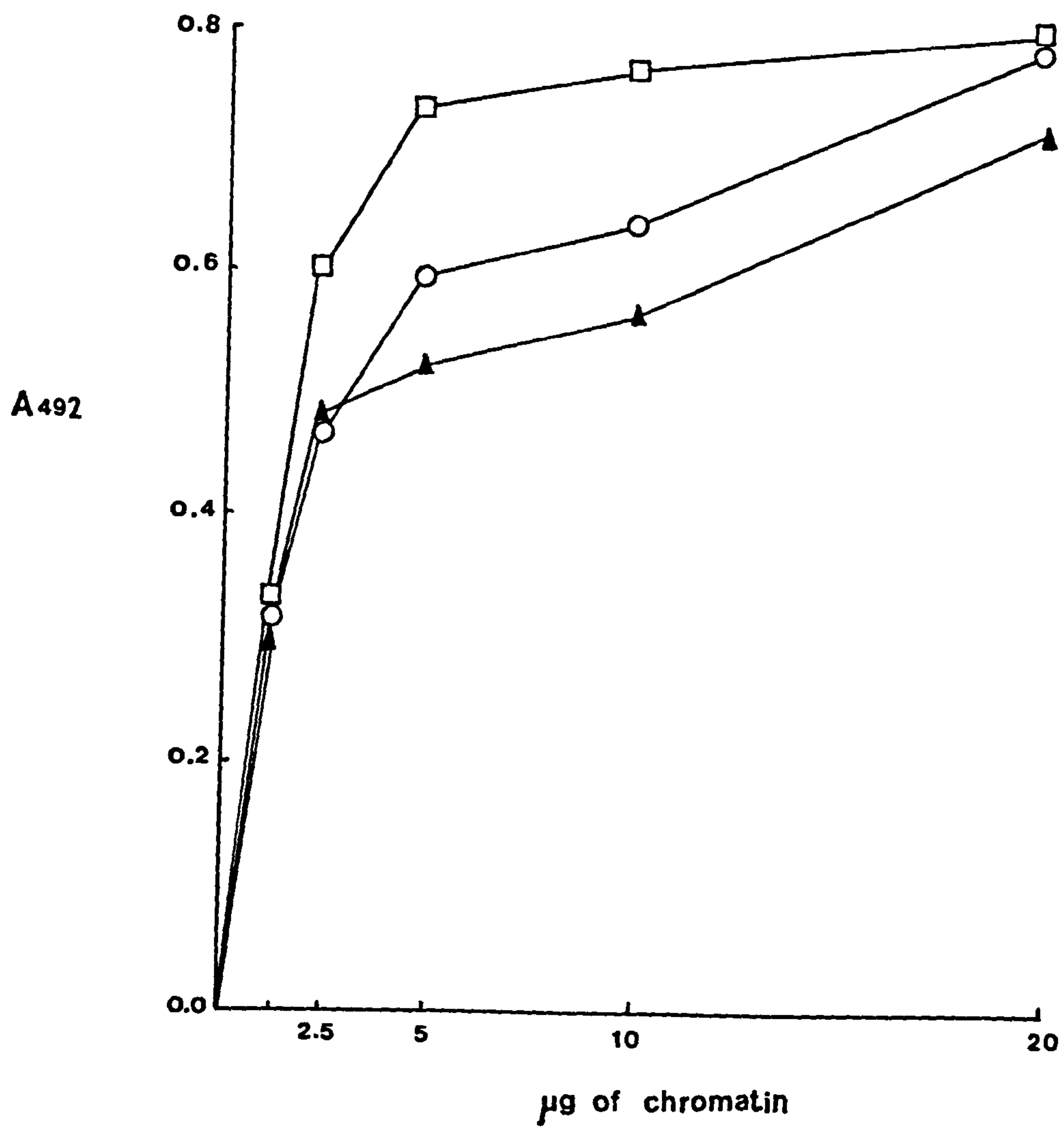


Figure 4.3.6      ELISA of HNo-G7 with Human Chromatins

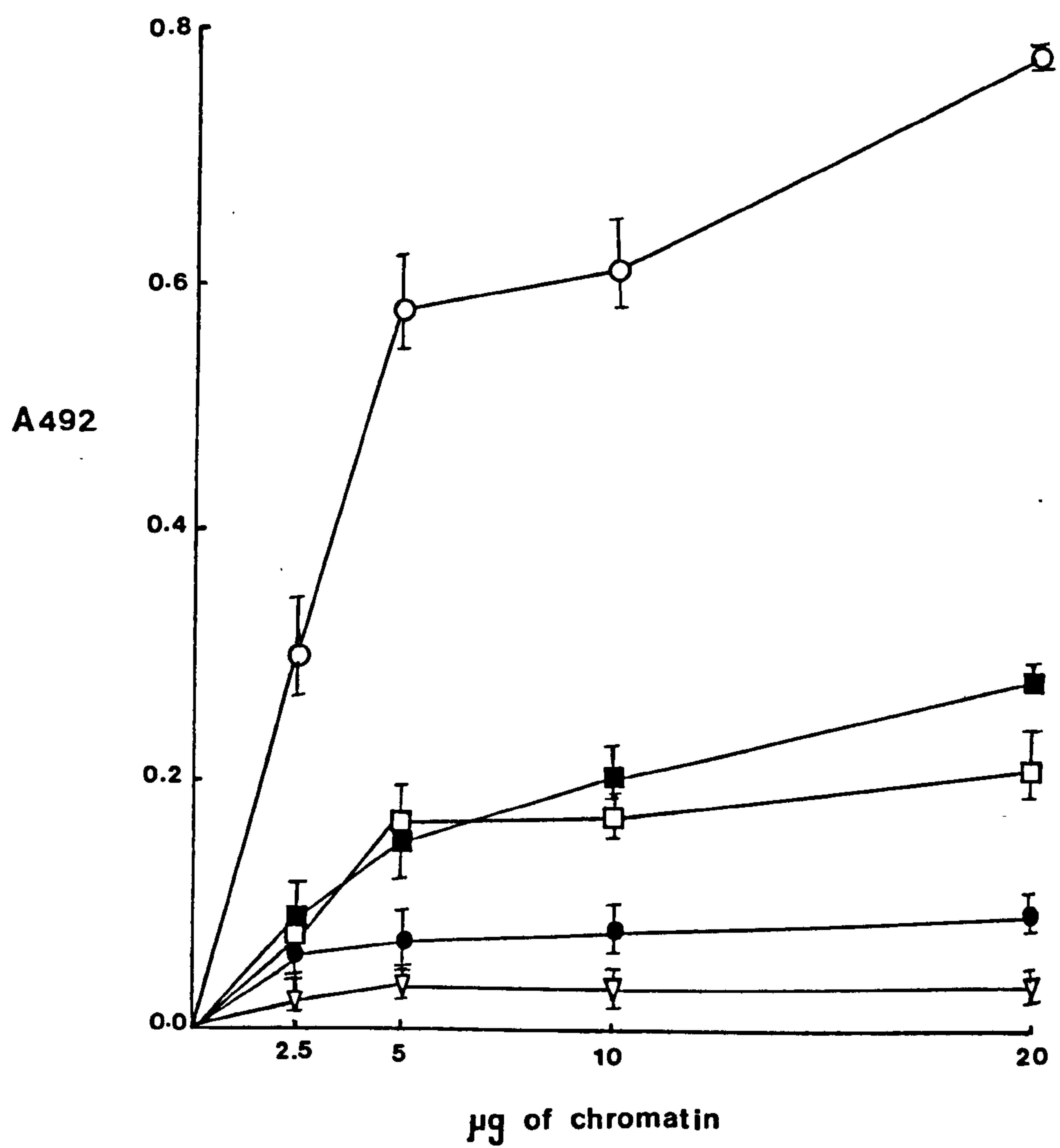
Enzyme linked immunosorbent assay of HNo-G7 (1/20)  
antibody reacted with different concentrations of various  
human chromatins. Antibody and antigen controls were  
subtracted. All chromatins measured as total protein  
content and DNA estimated as total DNA content.

- HeLa chromatin
- Chang liver
- H.Ep-2
- HT1080
- ▽ DNA

Antibody control was 0.07 A<sub>492</sub> unit while the antigen controls were as follows:

HeLa chromatin	: 0.147 A <sub>492</sub> unit
Chang liver	: 0.16 A <sub>492</sub> unit
H.Ep-2	: 0.142 A <sub>492</sub> unit
HT1080	: 0.145 A <sub>492</sub> unit
HeLa DNA	: 0.107 A <sub>492</sub> unit





from each sample was then added to a fixed amount of HeLa chromatin immobilised on an ELISA plate. The results are shown in Figure 4.3.7a. Much of the reactive antibody was absorbed by the HeLa chromatin while H.Ep-2 and Chang liver chromatins exhibited lower absorption profiles. The data are shown with greater clarification, replotted as a percentage in Figure 4.3.7b. It can be seen that HeLa chromatin at a concentration of 20µg/50µl of antibody (1/20) was able to bind almost 80% of the reactive antibody while H.Ep-2 and Chang liver chromatins were only able to bind 35% and 25% at the same concentrations. The data thus appear to indicate that the antigenic determinant is either present at lower concentrations or less accessible in the chromatins of the two other epithelial cell lines.

The HNo-G7 monoclonal antibody reacted variably with subcellular preparations from HeLa cells (Fig. 4.3.8). Chromatin, cytoplasmic, nucleolar and 2M salt soluble extracts of chromatin were allowed to react with the monoclonal antibody on ELISA, using fixed amounts of each preparation (10µg protein/well) bound to the plate. The chromatin preparation again demonstrated high reactivity with HNo-G7 and considerable but lesser activity was also present in cytoplasmic and nucleolar preparations. The 2M salt-soluble chromatin, which had been extensively dialysed against PBS buffer, failed to show any activity. As this preparation contains large amounts of histones these results indicate that the antigen is unlikely to be a histone.

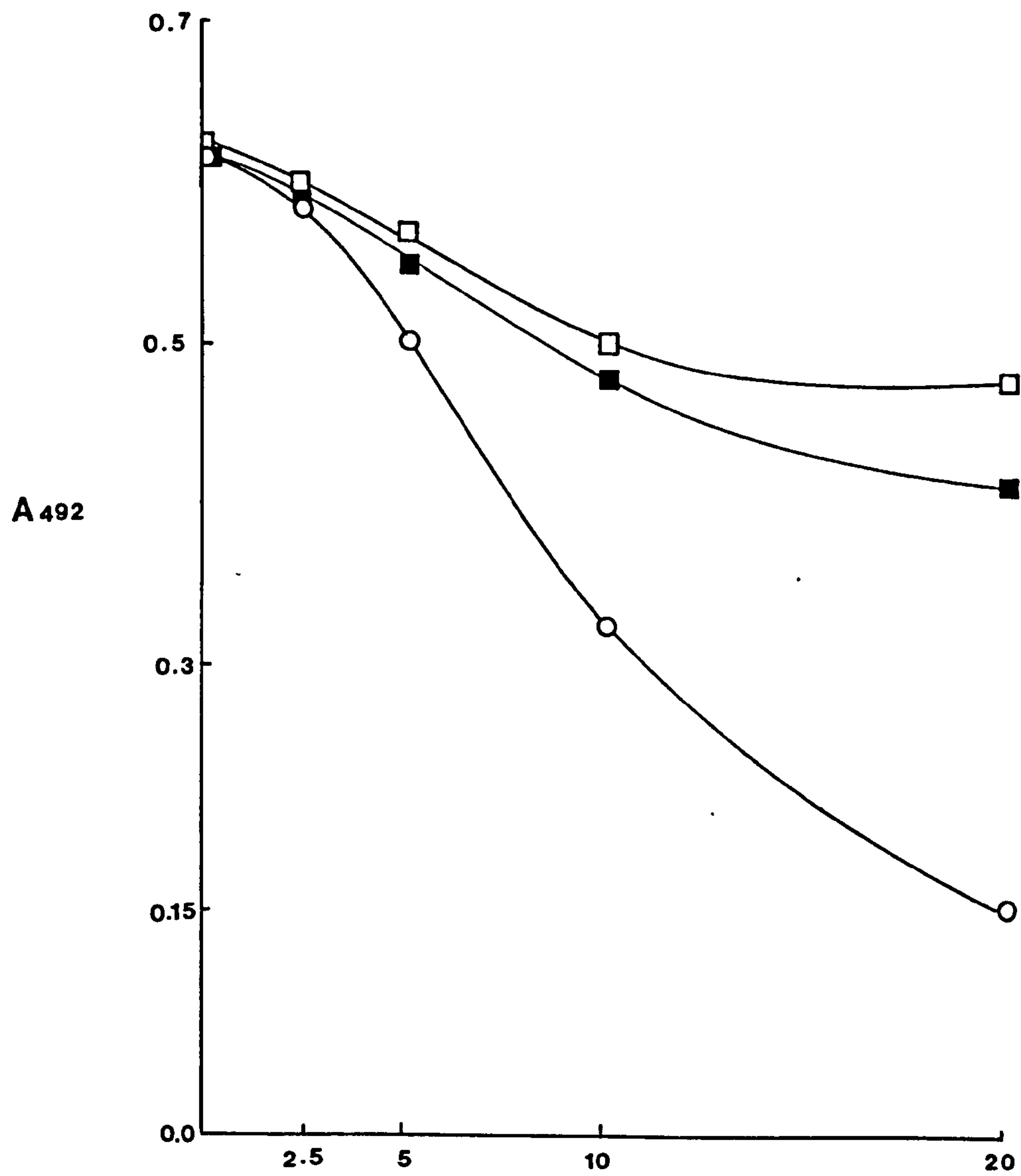
In order to confirm that the qualitative difference between the different subcellular fractions does not reflect differential binding of the proteins to the ELISA plate, a competition assay was performed in a manner similar to the assay used for Figure 4.3.7a and b. The results are shown in Figures 4.3.9a and b. It can be seen that HeLa chromatin competed very effectively against itself as would be expected with 80% of



Figure 4.3.7a    Competition ELISA of HNo-G7 with Human Chromatins

Enzyme linked immunoassay of HNo-G7 (1/20) absorbed with various concentration of human chromatins. The supernatants (50 $\mu$ l) after this incubation were allowed to react with constant amount of HeLa chromatin immobilised on an ELISA plate (section 3.4.5).

- HeLa
- H.Ep-2
- Chang liver



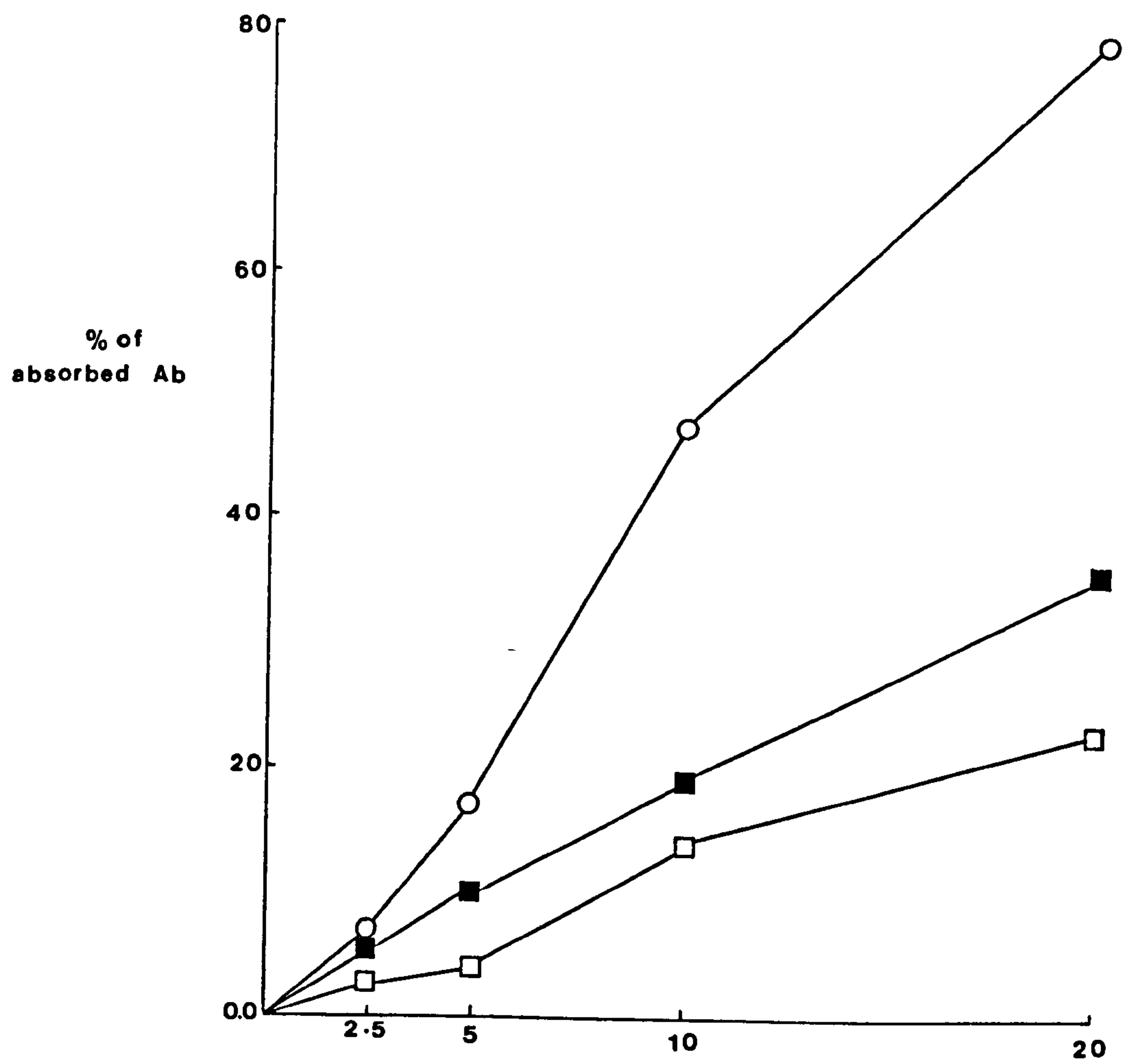
( $\mu\text{g}/50\mu\text{l}$ ) of various chromatin competed with  
HeLa Chromatin for the binding of HNo-G7



Figure 4.3.7b     Percentage of Absorbed HNo-G7 Used in Competition ELISA  
of Human Chromatins

The amount of the remaining reactive antibody (HNo-G7), after absorption with the chromatins described in 4.3.7a, expressed in percentage of total free HNo-G7 reacted with the immobilised HeLa chromatin.

- HeLa
- Chang liver
- H.Ep-2



( $\mu\text{g}/50\mu\text{l}$ ) of the competed Chromatins described  
in Figure 4.3.7a



Figure 4.3.8      ELISA of HNo-G7 with HeLa Subfractions

Enzyme linked immunoassay of different concentrations of the HNo-G7 antibody reacted with fixed amount of HeLa cell subcellular fractions (10<sup>protein</sup>µg/well) immobilised on an ELISA plate. Antibody and antigen controls were subtracted.

- HeLa chromatin
- Cytoplasmic preparation
- ▲ Nucleolar preparation
- 2M salt-soluble chromatin (Histones)

(the soluble protein was dialysed against PBS and the precipitate resuspended in the coating buffer as for the other preparations).

Antibody control was 0.05 A<sub>492</sub> unit and the antigens controls were as follows:

HeLa chromatin	:	0.14 A <sub>492</sub> unit
Cytoplasmic preparation	:	0.16 A <sub>492</sub> unit
nucleolar preparation	:	0.16 A <sub>492</sub> unit
Histone-rich fraction	:	0.103 A <sub>492</sub> unit

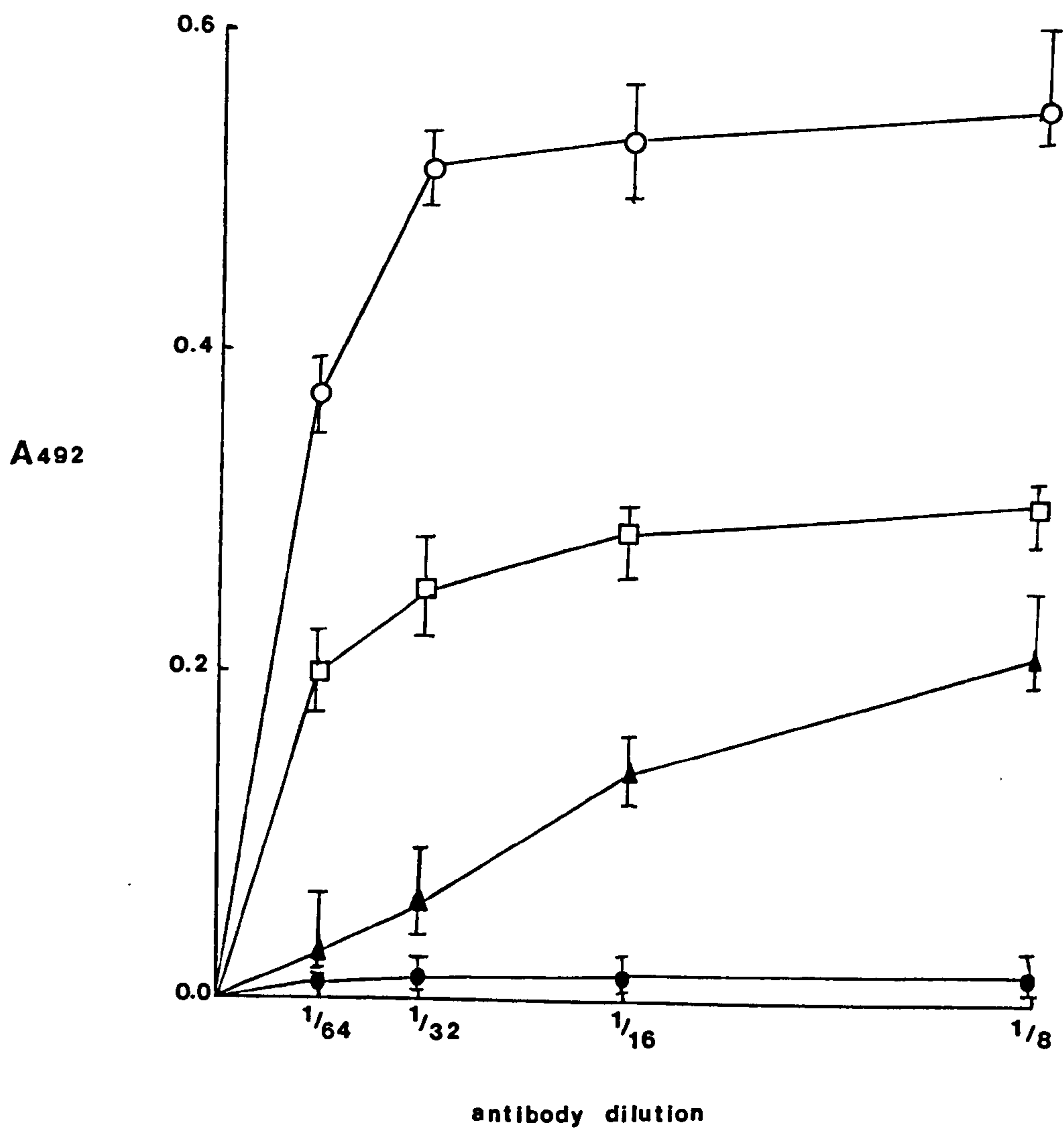
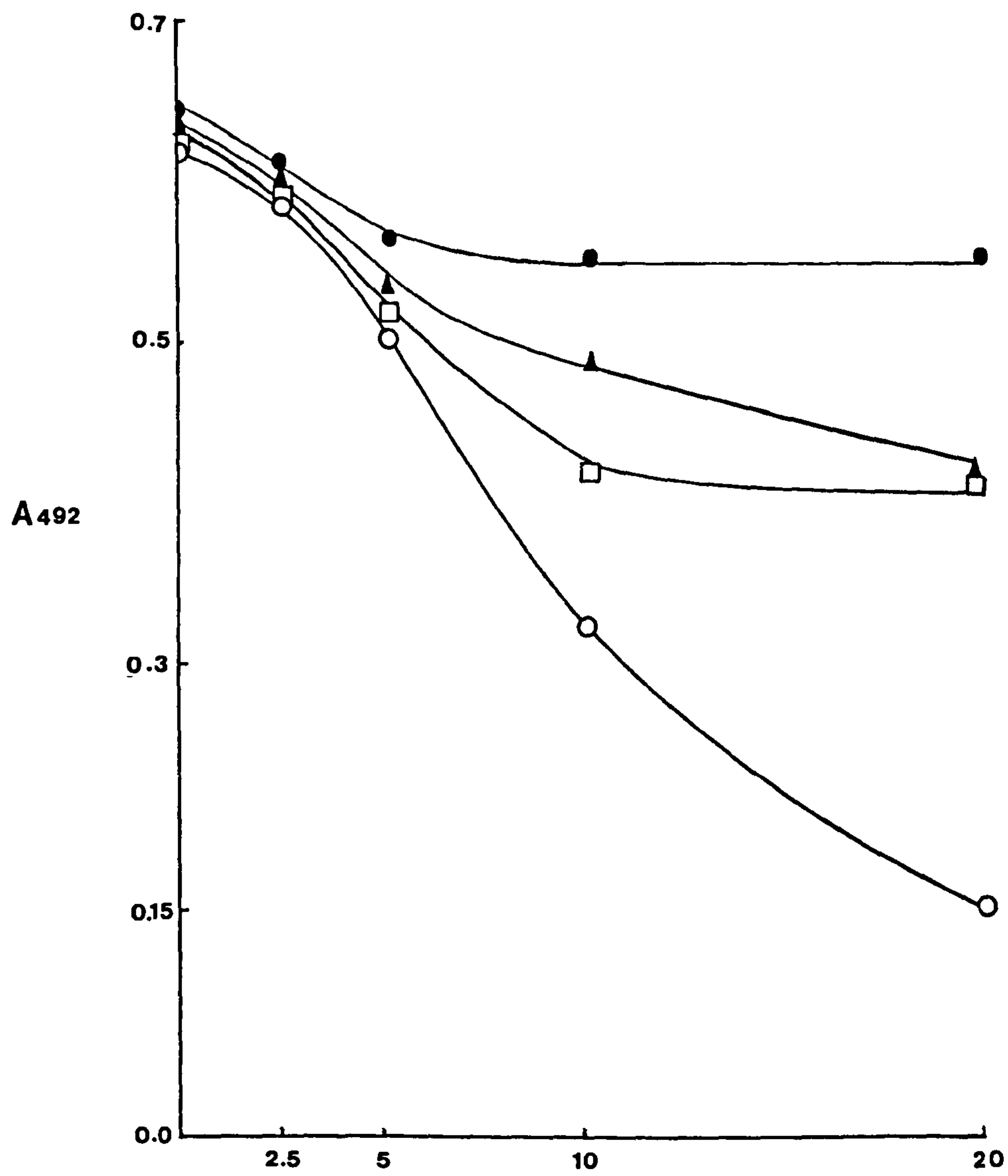




Figure 4.3.9a     Competition ELISA of HNo-G7 with HeLa Subfractions

Enzyme linked immunoassay of HNo-G7 (1/20) absorbed by various concentrations of HeLa subcellular fractions. The supernatant of the mixture was allowed to react with HeLa chromatin immobilised (10µg/well) on an ELISA plate. Antibody and antigen controls were subtracted.

- HeLa chromatin
- Cytoplasmic fraction
- ▲ Nucleolar preparation
- 2M salt-soluble chromatin (prepared as in Fig.4.3.8)



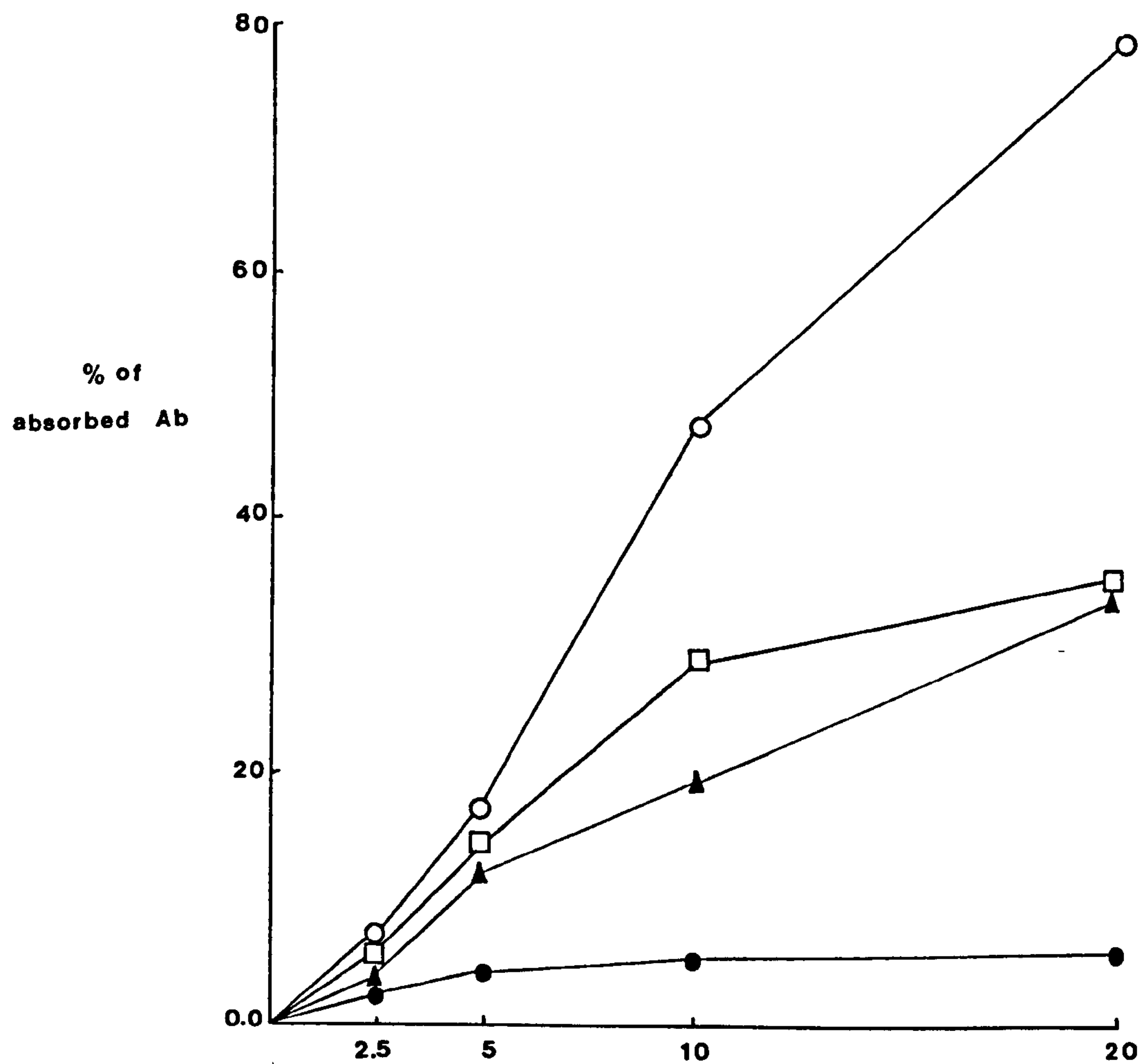
( $\mu\text{g}/50\mu\text{l}$ ) of protein competed with HeLa chromatin  
for the HNo-G7 antibody binding sites



Figure 4.3.9b    Percentage of Absorbed HNo-G7 Used in Competition ELISA  
of HeLa Subfractions

The amount of HNo-G7 antibody absorbed by different concentrations of HeLa cell subcellular fractions expressed as a percentage of free HNo-G7 reacted with immobilised HeLa chromatin on an ELISA plate.

- Chromatin
- Cytoplasmic preparation
- ▲ Nucleolar fraction
- Histone-rich fraction



( $\mu\text{g}/50\mu\text{l}$ ) of protein competed with HeLa chromatin  
described in Figure 4.3.9a



the antibody being absorbed at a concentration of 20 $\mu$ g of protein per 50 $\mu$ l of HNo-G7 (1/20). Both cytoplasmic and nucleolar preparations were able to absorb 33% of the HNo-G7 immunoactivity at the same protein concentration.

The antigen was further characterised by treatment with a variety of enzymes before reaction with the monoclonal antibody. Figure 4.3.10 shows that antigenic activity was unimpaired by treatment with excess of either DNAase I or RNAase A, indicating that the antigen itself is unlikely to be DNA or RNA and that it has no apparent conformational dependence on DNA or RNA for activity. Trypsin treatment, on the other hand, totally abolished the immunoreactivity indicating that the antigen is likely to be a protein or an epitope dependent on intact protein for its reaction with antigen.

The subcellular localisation of the antigen was further investigated by immunofluorescent staining of HeLa cells grown and fixed on coverslips. The cells were allowed to react with the monoclonal antibody followed by FITC conjugated rabbit anti-mouse IgG. The immunofluorescence is clearly concentrated in the nucleoli (Fig. 4.3.11) and some less intense staining is also evident in the cytoplasm and at the nuclear boundary (4.3.11). The cytoplasmic staining was less intense than the nucleolar one, and this is shown more clearly at high magnification (Fig. 4.3.11b & c). A control sample (in which tissue culture medium replaced the monoclonal antibody) showed very little staining (Fig. 4.3.12).

In order to investigate whether the antigen was present in metaphase chromosomes, and in particular on those with nucleolar organiser regions, a metaphase chromosome spread was prepared as described in sections 3.7.7 and 3.7.8 and reacted with the monoclonal antibody in the same manner as the interphase cells. The chromosomes

Figure 4.3.10    ELISA of HNo-G7 with Enzymes-Treated HeLa Chromatin

Enzyme immunoassay of monoclonal antibody (HNo-G7) reacted with different concentrations of HeLa chromatin and enzyme-treated chromatin. The enzyme concentrations were in excess as described in section 3.4.6. Antibody and antigen controls were subtracted.

- HeLa chromatin untreated
- DNAase I-treated (500µg/ml) chromatin
- ▼ RNAase A-treated (200µg/ml) chromatin
- Trypsin-treated HeLa (200µg/ml) chromatin

No attempt was made to estimate the extent of digestion of DNA or RNA. However the nucleases were used in amounts which were in substantial excess (as judged by specific activity) to those required to digest all the nucleic acid present.



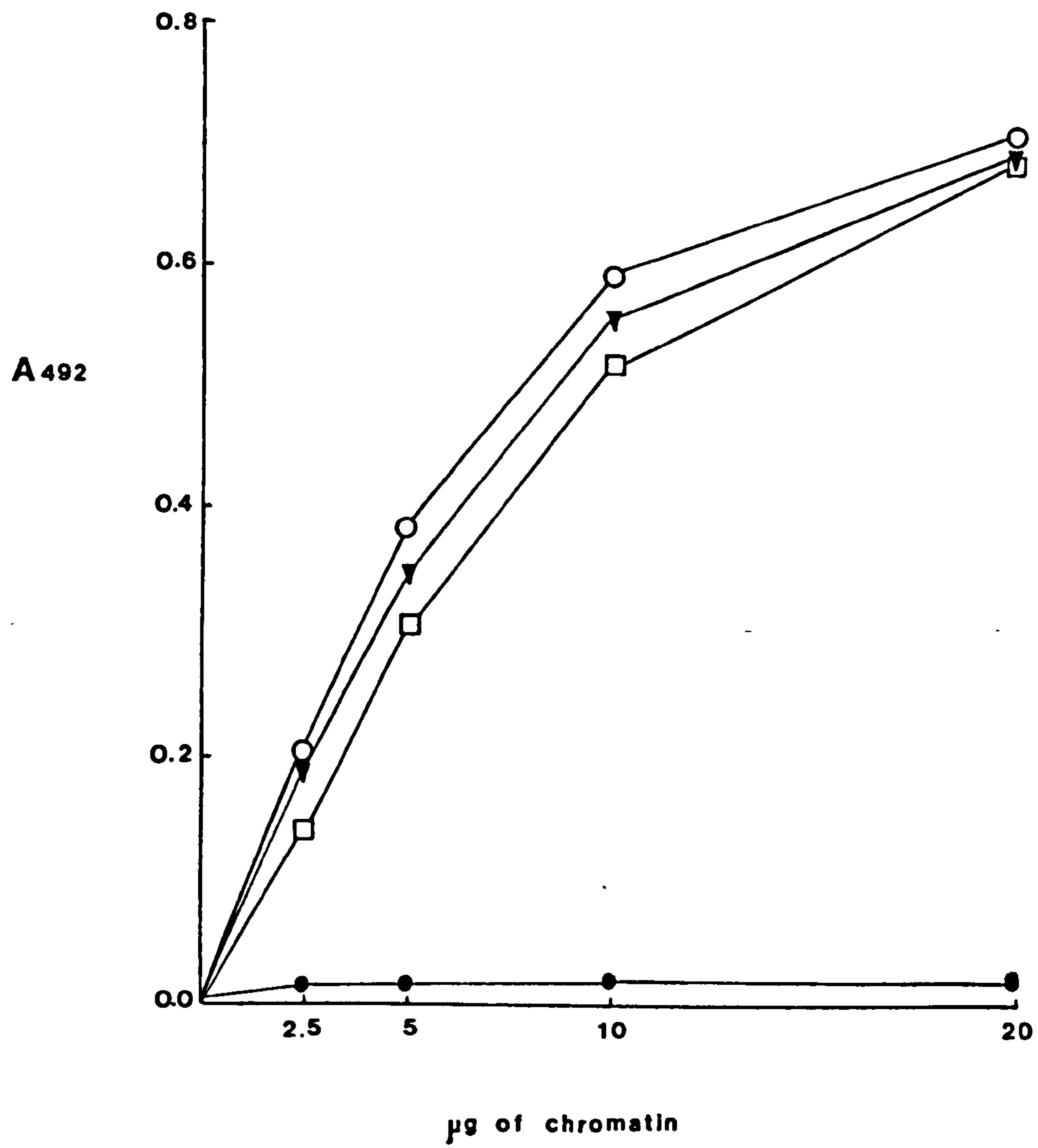


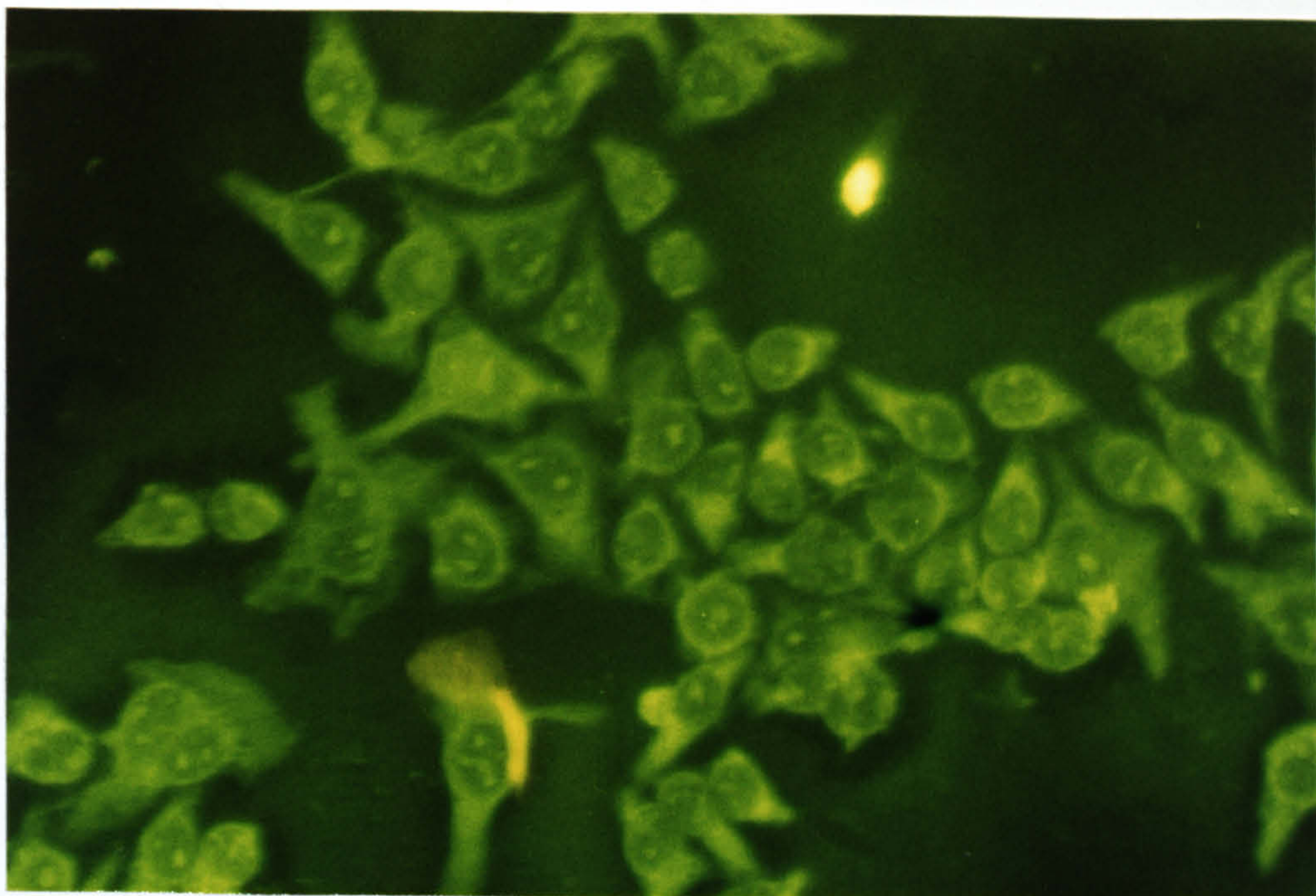
Figure 4.3.11     FITC-staining of HeLa Cells Reacted with HNo-G7 and 653  
Culture Supernatants

Indirect immunofluorescent staining of HeLa cells reacted  
with HNo-G7 antibody (1/10).

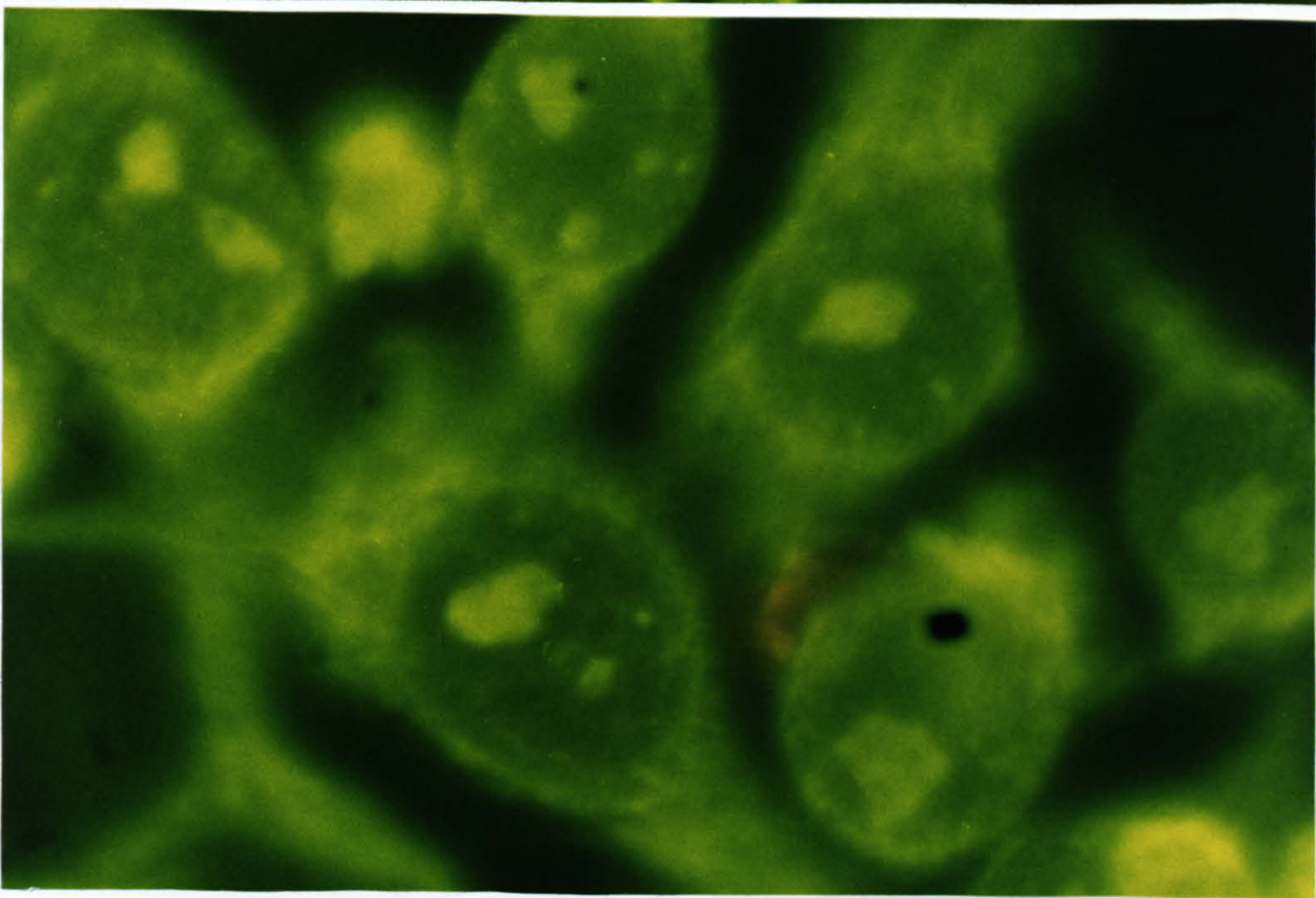
- a)        Low magnification (X500)
- b & c) High magnification (X2000)
- d)        Black and white photograph
  - 1.    FITC stained cells (X375)
  - 2.    Phase contrast of 1 (X375)
  - 3.    FITC stained cells (X500)
  - 4.    FITC stained cells (X2000)
  - 5.    FITC-control of HeLa cells reacted with  
      Culture-Supernatant from 653 myeloma cells.



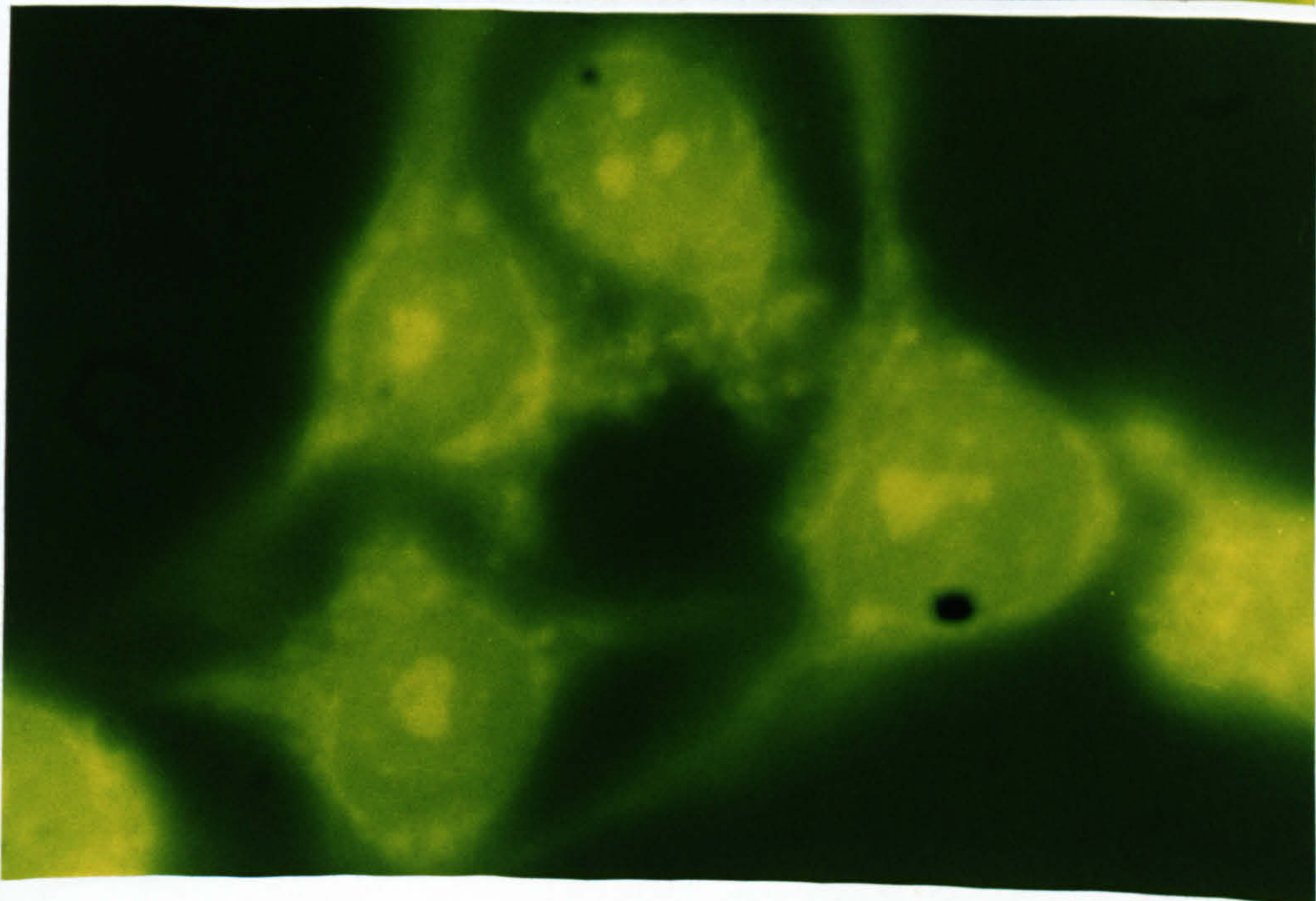
a



b

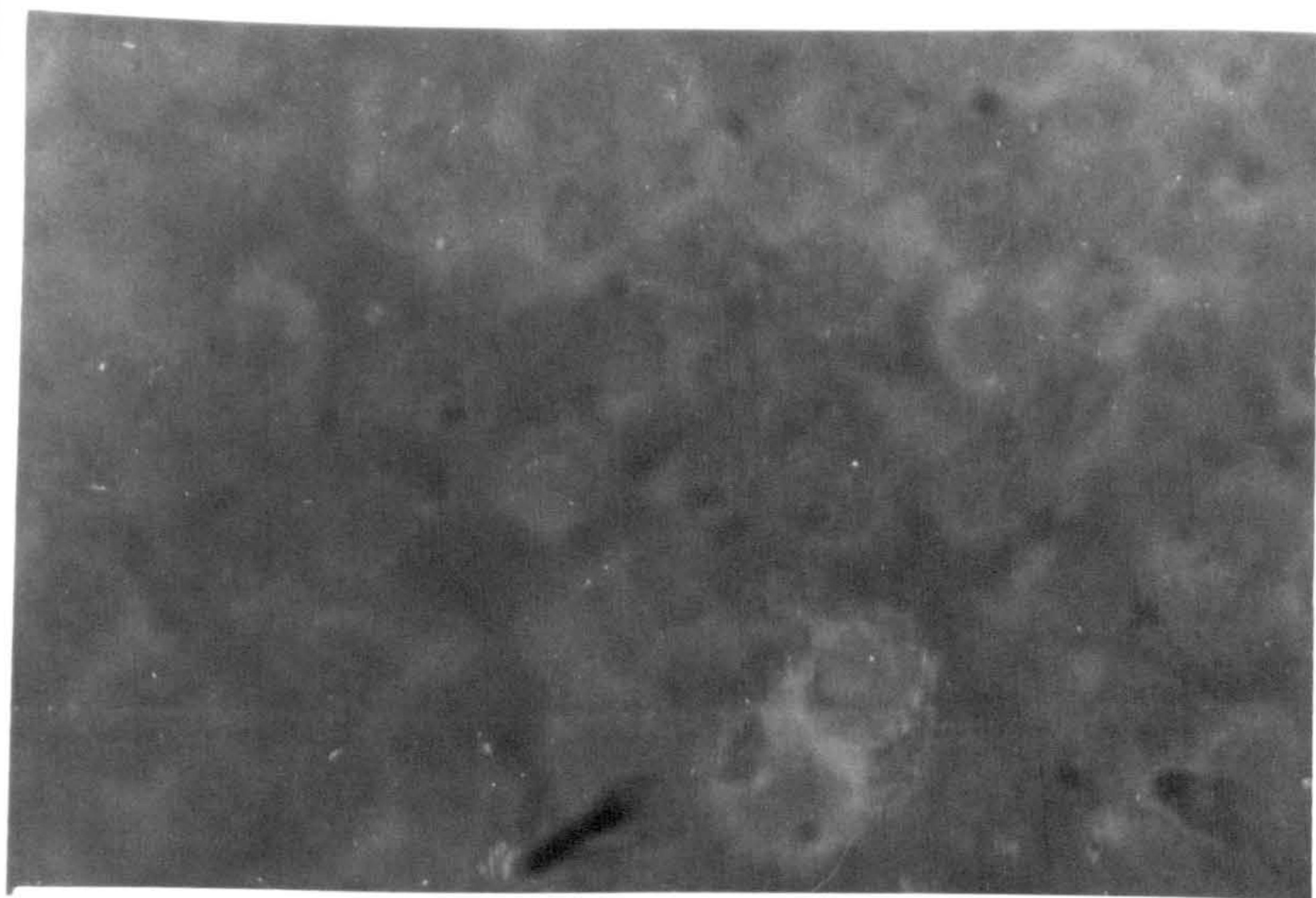
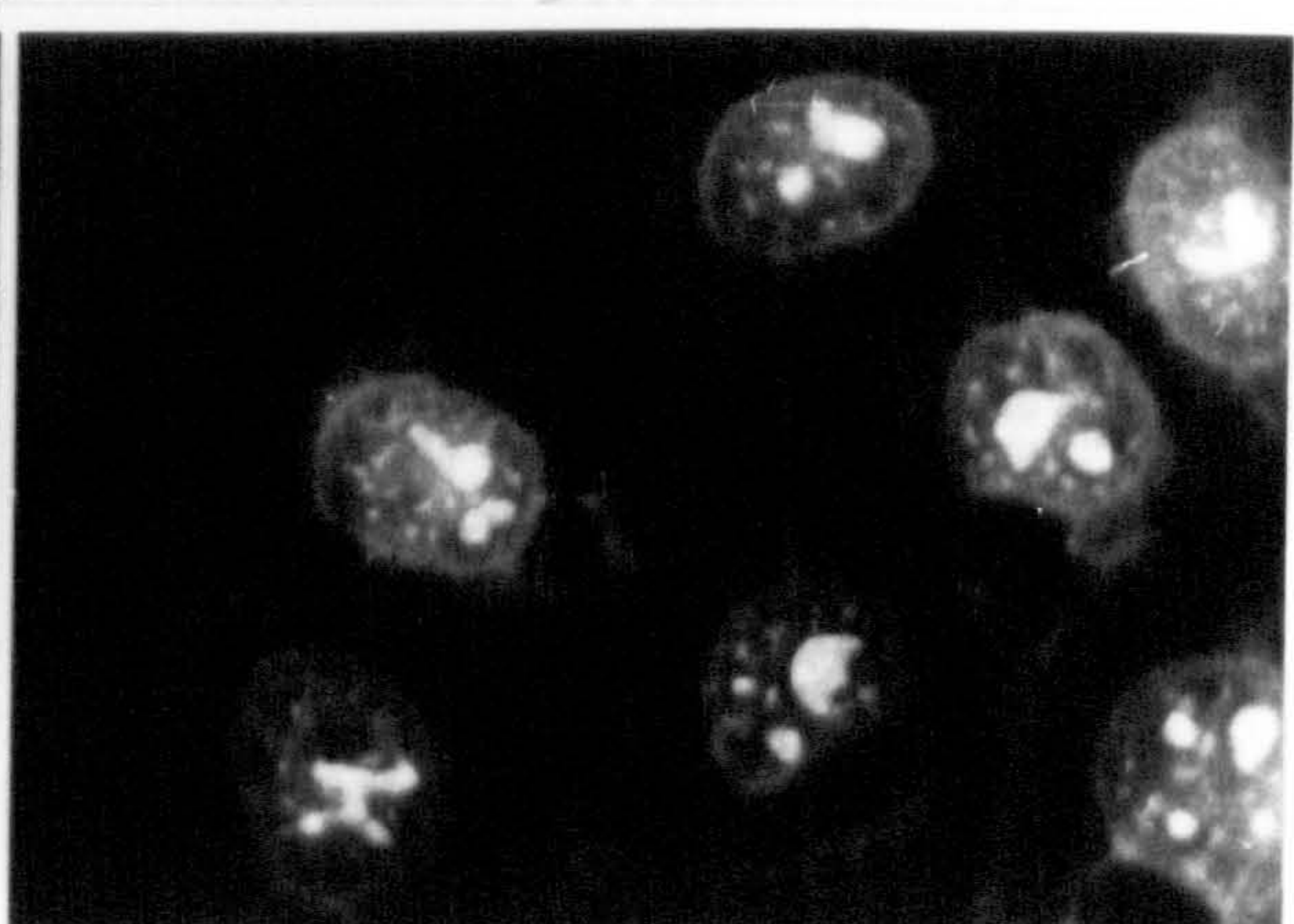
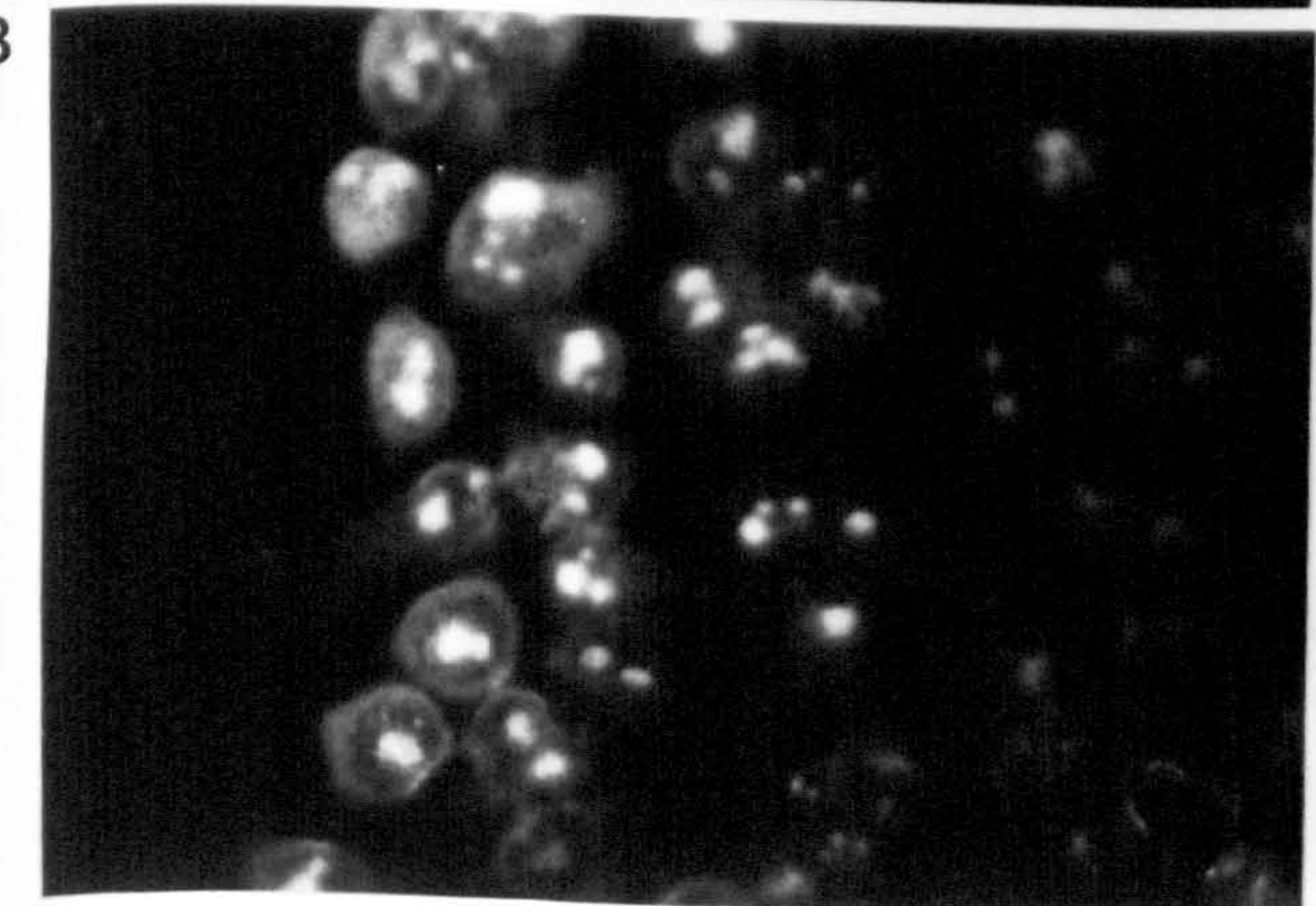
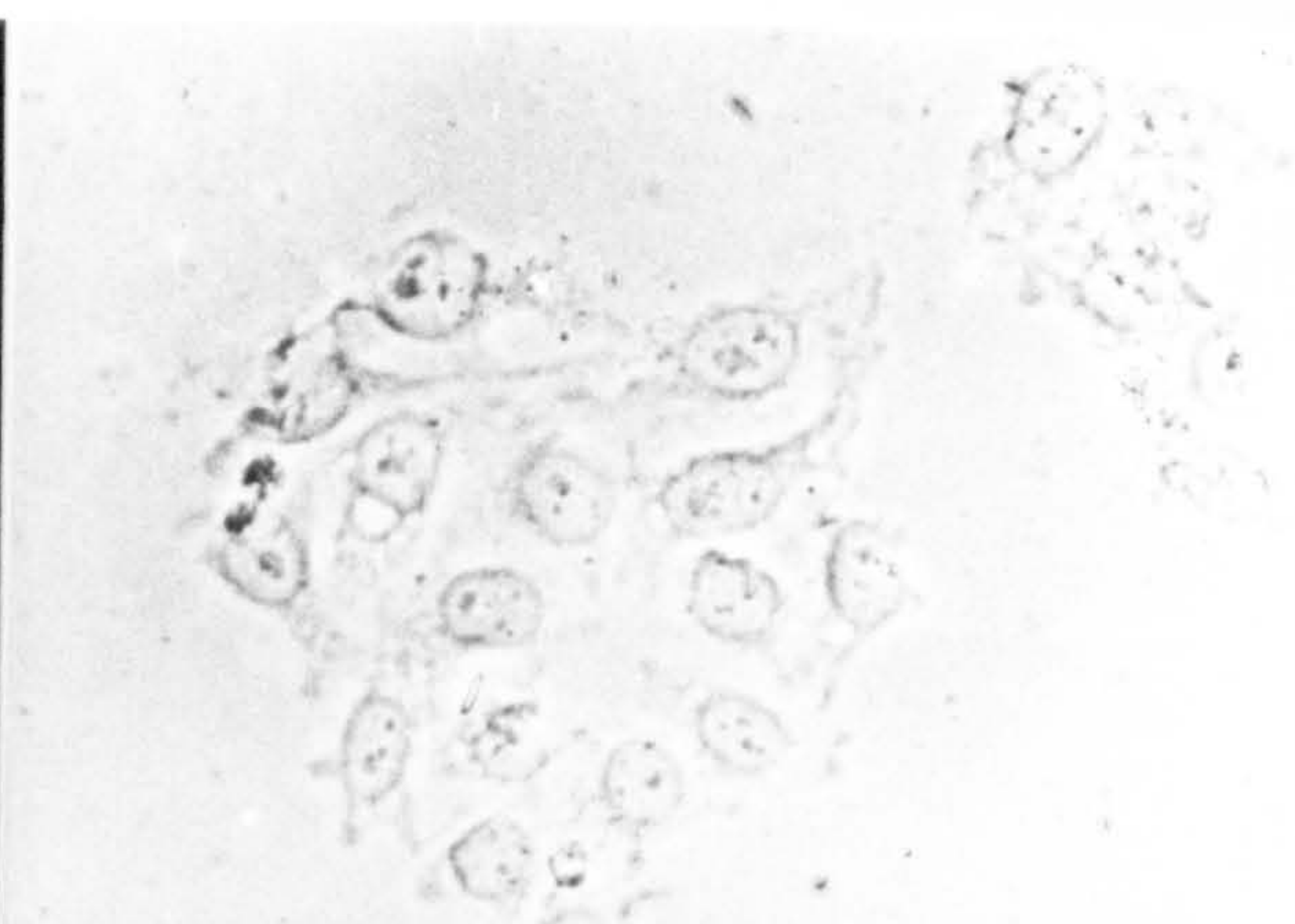
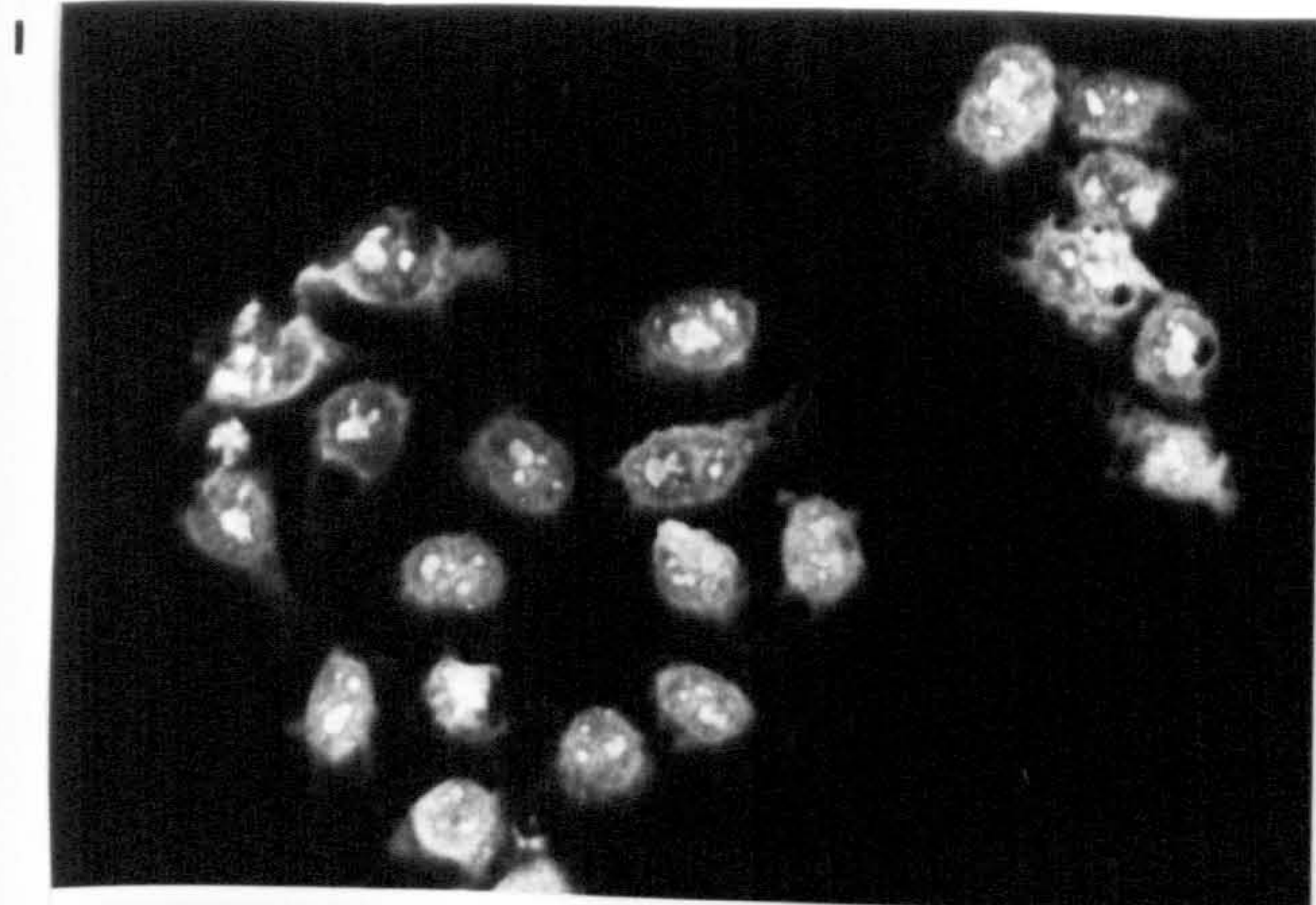


c





d



5



Figure 4.3.12    FITC-control of HeLa Cells Reacted with RPMI-FCS

Indirect immunofluorescent staining of HeLa cells treated with RPMI-FCS instead of the first antibody (HNo-G7) followed by anti-mouse IgG- FITC-conjugated second antibody (X500).



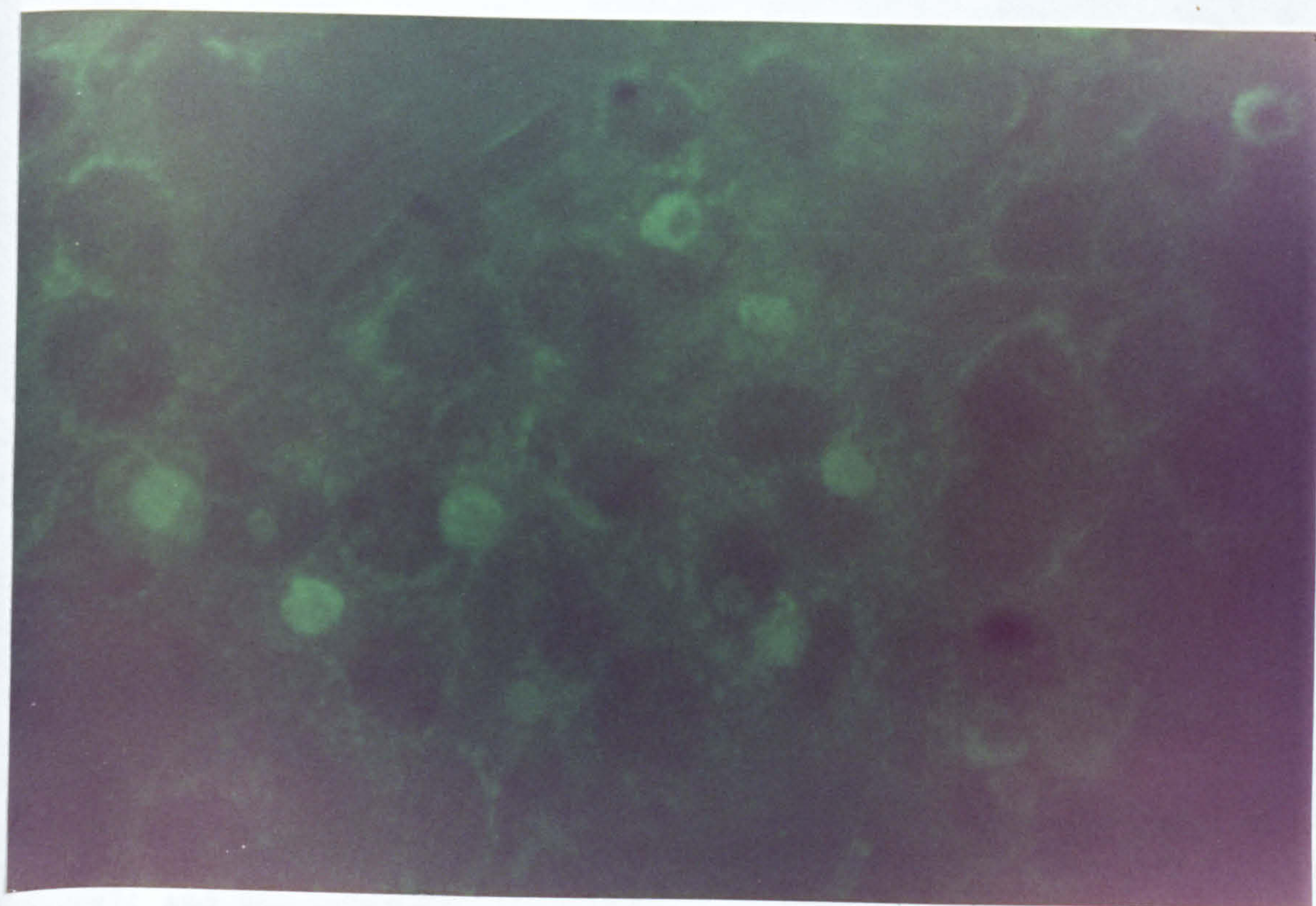


Fig. 1

Section of the

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were also separately stained with Giemsa to ensure that the quality of the spread was adequate (Fig. 4.3.13). No fluorescence was detected in these preparations with the monoclonal antibody indicating that either the antigen is not present in detectable quantity or it is not accessible in metaphase chromosomes.

In view of the fact that neither DNA nor RNA were required for antigenic activity, experiments designed to further characterise the protein component of the antigen by immunoblotting were then undertaken. Immunoblotting of a monoclonal antibody presents particular technical problems as it is likely that only a single epitope will be present on each molecule of the antigenic protein and the signal is therefore likely to be extremely weak in comparison to that from a polyclonal antiserum. It is therefore important to have as high a concentration of specific protein antigen as possible on the nitrocellulose paper. Dehistonised chromatin was consequently chosen for this procedure as more of the relevant material may be loaded on the gel in the absence of the histones. Two different preparations of dehistonised chromatins were employed. The first was extracted with 2M salt and the second with sulfuric acid by the method of Bhorjee and Pederson (1973) (section 3.1.4). The result is shown in Figure 4.3.14a using the immunoperoxidase (section 3.5.4.1) detecting procedure. the monoclonal antibody used was collected from culture supernatant after growing the cells to exhaustion. Figure 4.3.14b also illustrates the reaction of monoclonal HNo-G7 with 2M salt-dehistonised chromatin resolved on an SDS-gel electrophoresis and blotted onto a nitrocellulose paper. The reaction was monitored and detected by a second antibody (rabbit anti-mouse IgM, H+L) followed by labelled  $^{125}\text{I}$ -protein A. In both Figures 4.3.14a and 4.3.14b, a reactive antigen of approximate molecular weight 44,500-45,000 was observed. This reactive antigen could be seen in both the acid-extracted and the 2M salt-extracted

Figure 4.3.13    Giemsa Staining of HeLa Metaphase Chromosomes

HeLa chromosome spread stained with Giemsa staining as described in sections 3.7.7 and 3.7.8 (X2000).



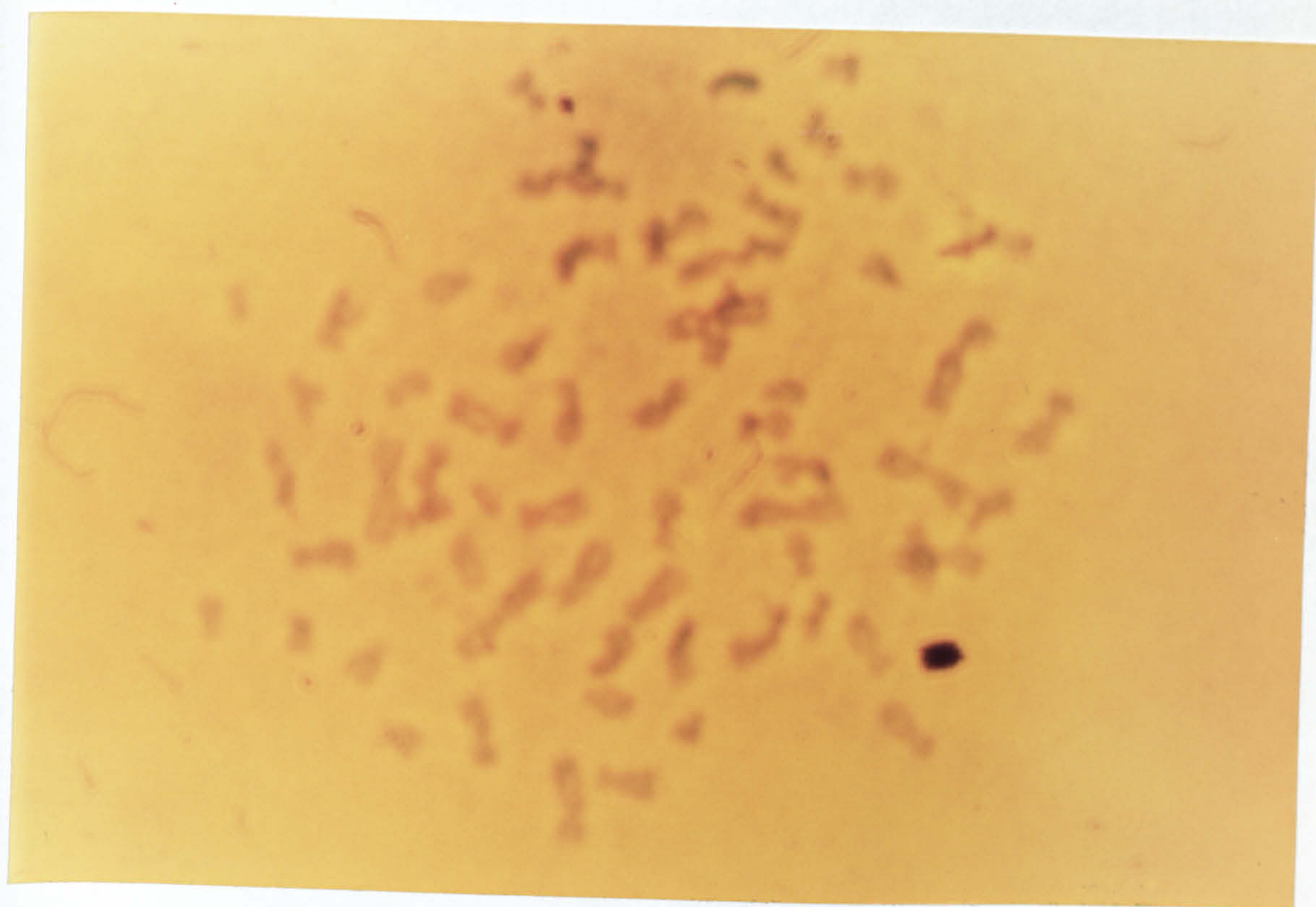


Figure 4.3.14a Immunoblot of HNo-G7 Reacted with HeLa Dehistonised  
Chromatins

Protein immunoblot of the HNo-G7 antibody reacted with nonhistone chromosomal fraction prepared by sulfuric acid extraction (lane c) or 2M salt-extraction of the HeLa chromatin (lane d), resolved on a 10% Laemmli gel. Detection of antibody reaction was by the immunoperoxidase method (section 3.5.4.1). Lane (a) represents the amido black-stained acid extracted - nonhistone proteins and lane (b) is the molecular weight protein marker (from top: phosphorylase-b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100).



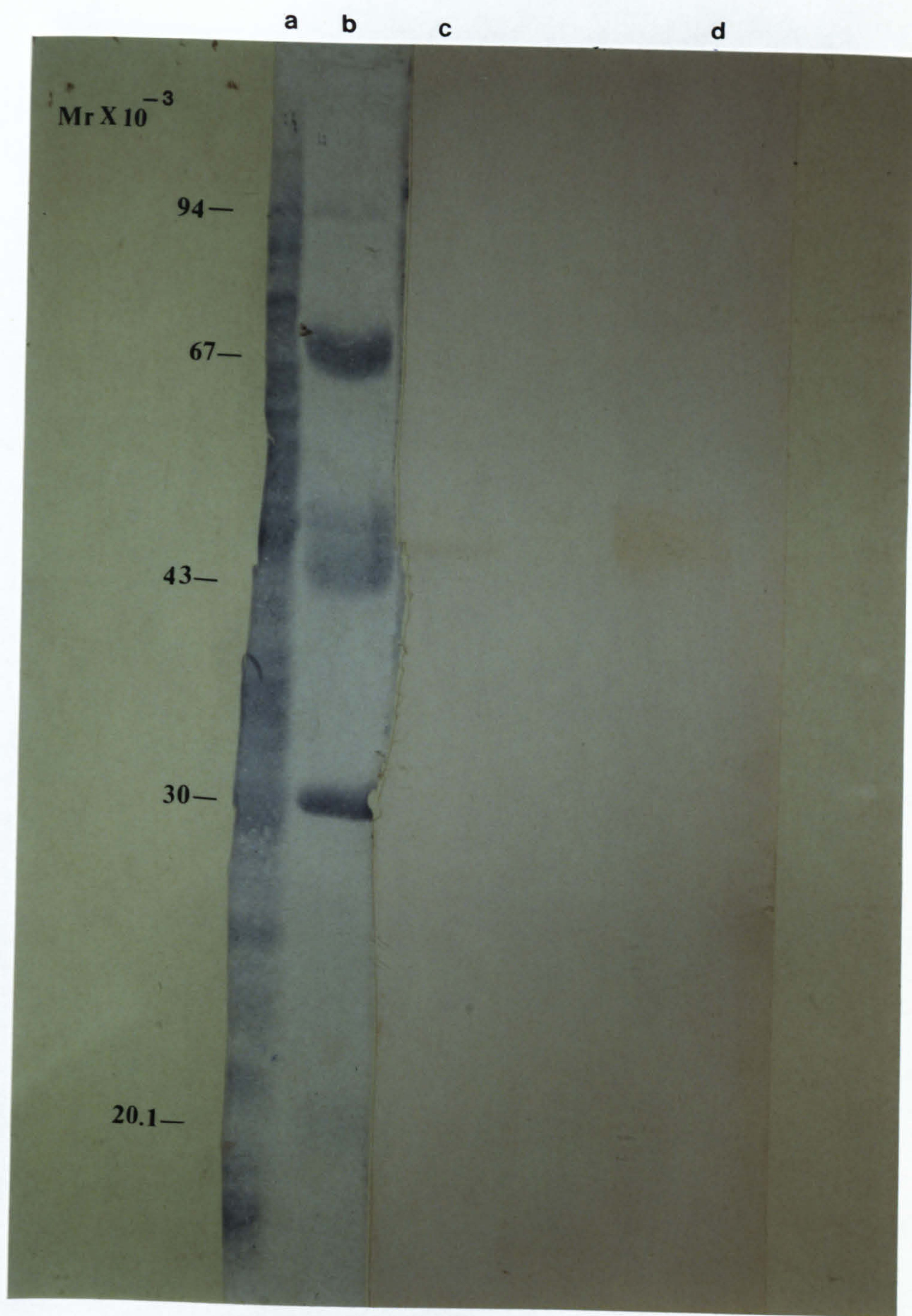




Figure 4.3.14b Autoradiograph of HNo-G7 Reacted with HeLa Dehistonised  
Chromatins

Protein immunoblot of HNo-G7 antibody (concentrated X3 by PEG 6,000) reacted with 2M salt-extracted nonhistone chromosomal protein fraction (from HeLa cells), resolved on 8.5% Laemmli gel and blotted onto nitrocellulose paper. The reaction was detected by the protein A method as described in section 3.5.4.3.

- Lane (1) An autoradiogram of the antibody reaction detected by  $^{125}\text{I}$ -protein A. (Eight hours exposure)
- Lane (2) Amido black stained 2M salt extracted chromatin blotted on nitrocellulose paper.
- Lane (3) Protein marker (from top: phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100;  $\alpha$ -lactalbumin, 14,400).

Note: Staining of the blot was by 0.1% amido black in 2% acetic acid and destaining by 2% hot (90°C) acetic acid. This modification is to prevent any shrinkage of the blot (Du Bois & Rosen, 1983).



1 2 3

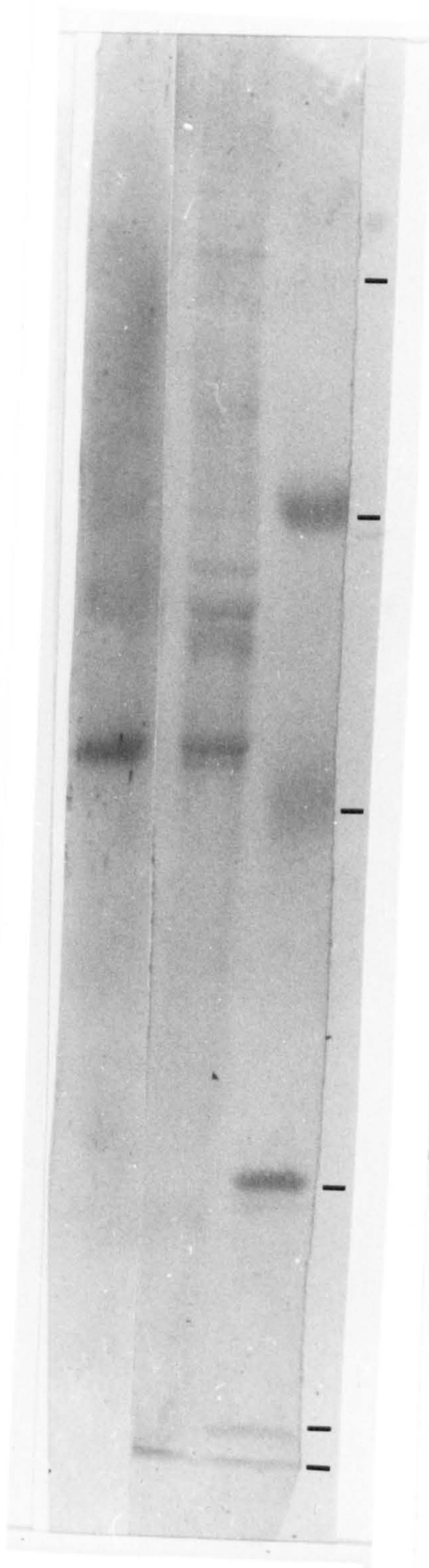


Figure 4.3.14c Autoradiograph of Control-Immunoblot

An autoradiograph of protein immunoblot of supernatant (concentrated X5) from an irrelevant IgM-Secreting hybridoma (anti-human thyroglobulin) reacted with the HeLa 2M salt-extracted nonhistone chromosomal protein fraction resolved on 8.5% Laemmli gel and blotted onto nitrocellulose paper. The reaction was detected by the protein A method. (Exposure was for 14 hours).





nonhistone chromosomal protein fractions. Normal mouse serum (from an unimmunised animal diluted 1/200) showed no reactivity with HeLa dehistonised chromatin nor did the supernatant from other IgM-secreting mouse hybridomas (irrelevant IgM) (Figure 4.3.14C).



## DISCUSSION

The HNo-G7 monoclonal antibody was clearly shown to be of the IgM class both by ELISA and internal radio-labelling. IgM producing hybridomas may be expected to be less predominant in a fusion from a hyperimmune animal since they are generally produced early in the immune response. The labelled antibody used in the selection assay was directed against IgG heavy and light chains so it may be expected to detect all isotypes because of its reaction with the light immunoglobulin chain. IgM antibodies have in general lower intrinsic affinity for the antigen than IgG antibodies directed to the same epitope (Rodwell et al., 1983). However, their multivalence can lead to an experimentally high affinity in selection procedures. Additionally, some antigens such as the ABO blood grouping antigens, appear to elicit an isotype specific IgM response (Taussig, 1979). With a single sample, it is not possible to say whether the fact that HNo-G7 is an IgM reflects an isotype preference of the antigen, a phenomenon related to the immunisation and selection procedure, or chance.

The internal radio-labelling of the monoclonal antibody provides information relating not only to its class but also to its monoclonal nature, since the hybridoma cell line clearly does not synthesise any other isotype. The possibility that the antibody is not monoclonal but is the product of two different co-existing hybridomas both secreting IgM cannot be totally discounted but it is highly unlikely since the hybridoma cell line has been subcloned repeatedly at 0.5 cell/well and antibody secretion and titre have remained constant over a period over 18 months. In mixed cultures this is almost never true. Attempts to obtain final proof of the true monoclonal nature of the antibody by isoelectric focussing were abandoned because of the extreme difficulty of introducing such a large molecule into the gel medium.

The preliminary characterisation of the binding of the antigen to the ELISA plate and the chequerboard assay are extremely important in the design of any screening system for a monoclonal antibody. It is important to be able to bind as much chromatin as possible to the plate in order to detect positive fusion clones as early as possible so that they may be cloned before overgrowth by non-secreting cells can occur. However, it is also vital that the antigen is bound stably to the plate and is not able to leach off during washing procedures (Lehtonen & Viljanen, 1980) since leached antigen could react with the monoclonal antibody with both being removed during a subsequent wash.

Early ELISA assays utilised high pH buffers for antigen binding (Engvall & Perlman, 1971). While more antigen may bind under these conditions, incubation with antibody is carried out at lower pH values and it is again vital, for the reasons described above, that antigen bound at high pH does not desorb during subsequent incubations at lower pH values. In addition, the pH dependence of the antibody - antigen reaction itself can affect the screening procedure strongly since some monoclonal antibodies exhibit very sharp pH profiles (Fraser et al., 1982). The experiments described in Figures 4.3.3 and 4.3.4 indicate that pH effects are unlikely to affect either antigen binding or reaction with antibody under the routine assay conditions employed.

The actual amount of antigen bound to the plate can be readily determined with a pure protein antigen since it is possible to radiolabel such an antigen and determine the percentage of radioactivity bound at various protein concentrations. However, with chromatin as an antigen this is almost impossible since the multiple components may incorporate label differentially and also bind differentially. However, some indication may be obtained from the chequerboard assay since the absorbance at 492nm was comparable over the range of 2.5-20µg/well at fixed antibody concentration (Fig. 4.3.5). Accordingly, it is possible



to suggest that the antigen binding onto the ELISA plate is restricted above a concentration of 2.5 $\mu$ g/well.

Very small amounts of antigen (155ng/well) could be detected by the monoclonal antibody as shown by the chequerboard. This suggests either that the affinity of the antibody is extremely high, or that the protein is present in substantial amounts in chromatin. Variation of the antibody concentration over the dilution range of 1 to 1/32 at fixed antigen concentration also has minimal effect, indicating that at a concentration of 1/32 there is still sufficient antibody in the supernatants to saturate all the antigen molecules bound to the plate.

In principle, some indication of the antigen bound to the plate can be obtained from the competition ELISA assay where HeLa chromatin is effectively competed against itself. Thus again with a simple protein antigen and at limiting concentrations of antibody, the amount of protein competitor required to abolish all antibody reactivity may be regarded as a rough indication of the amount of protein actually bound to the plate. Even in this situation such a conclusion is suspect since the binding of proteins to ELISA plates causes some degree of denaturation (Berkowitz & Webert, 1981). With chromatin as antigen, any such conclusions become even less valid. Firstly, some components of chromatin may bind more readily to the plate than others and consequently the comparison of competing antigen in solution with plate bound test antigen is not possible. The antibody is an IgM with ten potential binding sites in solution and it is highly unlikely that all these could bind to antigen on the plate for obvious steric considerations. Additionally, the pieces of free competing chromatin may have multiple copies of antigen on them further confusing the results. The comparatively large amounts of antigen required for self competition with HeLa chromatin probably reflect the fact that the antibody is being used in excess as indicated by the chequerboard experiment and it is not

possible to use the data to give a meaningful estimate of the amount of antigen actually bound to the plate.

Many of the above considerations also apply to the use of conventional or competition ELISA, to compare different preparations of chromatin or subcellular fractions. However, on a comparative base of protein (or DNA) concentration the data from Figures 4.3.6 and 4.3.7 appear to indicate that the antigen is present in substantially greater amounts in HeLa chromatin than in the chromatin of other epithelial cell lines and that it is not detectable in a fibrosarcoma cell line. there are several possible interpretations of this result. The most likely is that the antigen is related to the metabolic activity of the nucleoli in some way and as this differs with different cell lines, so the amount of antigen also varies on a protein or DNA basis. Another possible interpretation is that the antigen is more accessible in HeLa chromatin than the other cell lines. There may also be an effect relating to the number of nucleoli (at least five pairs of chromosomes carry nucleolar organisers (Flavell & Martini, 1982)) which may be greater in HeLa cells which carry a large aneuploid chromosome complement (Adams et al., 1981). The lack of any apparent antigen in a fibrosarcoma cell line may indicate that the antigen has some preference for epithelial cells but this cannot be definitely ascertained until a wider panel of human cell lines has been tested.

With a monoclonal antibody the possibility that the antigen is an epitope which is present on different proteins in each case (for example a carbohydrate residue) can not be ruled out.

The use of conventional and competition ELISA assays to determine the subcellular localisation of the antigen carries even greater hazards of interpretation since, while competing chromatin all have similar physical characteristics (this is especially true comparing Chang liver and HeLa chromatin which are slightly different from H.Ep-2 chromatin as



illustrated in Figure 4.3.15), the competing antigens in subcellular localisation have very dissimilar physical properties. In addition, the preparation of the various components may result in substantial cross contamination. Within these limits, it is however possible to conclude from Figures 4.3.8 and 4.3.9 that the antigen is present in chromatin, nucleolus and cytoplasm and not detectable in the 2M NaCl-extracted fraction which contains the bulk of the histones. The comparatively large amount present in the cytoplasm on the protein basis is surprising. As the immunofluorescent staining (Fig. 4.3.11) is predominantly nucleolar, the comparatively small amount in the nucleoli is also unexpected. However, the nucleolar preparation consists mainly of condensed histones which compete with other proteins, including the antigen, on the basis of total protein estimation and it should be noted from Figure 4.3.9 that at high concentrations the nucleolar preparation competed almost as well as cytoplasm in the competition assay.

A general correlation with immunofluorescence may be made but it is quantitatively different. This difference may again be attributed to several factors. The physical nature of the antigen in the various preparations may affect its ability to bind to the plate or differentially affect the reaction of plate bound chromatin antigen with competitor in solution. It is also very possible that the antigen may move from one cell compartment to another during the preparative procedure as in the case described by Davis et al. (1978) who reported a nucleolar antigen which was preferentially localised in the nucleoli in immunocytochemistry but which appeared to be able to equilibrate between the nucleoplasm and the cytoplasm during isolation. Additionally, preparation of the nucleoli by the method of Bhorjee and Pederson (1973) which involves sonication may result in the loss of substantial amounts of antigen. It is also possible that the antigen may genuinely be present in substantial amounts in the cytoplasm as well as the nucleoli

Figure 4.3.15a Protein Blot of Human Chromatins

An amido black stained blot comparing three chromatin preparations from epithelial human cell lines. The blot stained by 0.1% amido black as described in Fig.4.3.14b.

Lane (1) Molecular weight marker: from top: bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor 20,100.

Lane (2) HeLa

Lane (3) H.EP-2

Lane (4) Chang liver



1 2 3 4

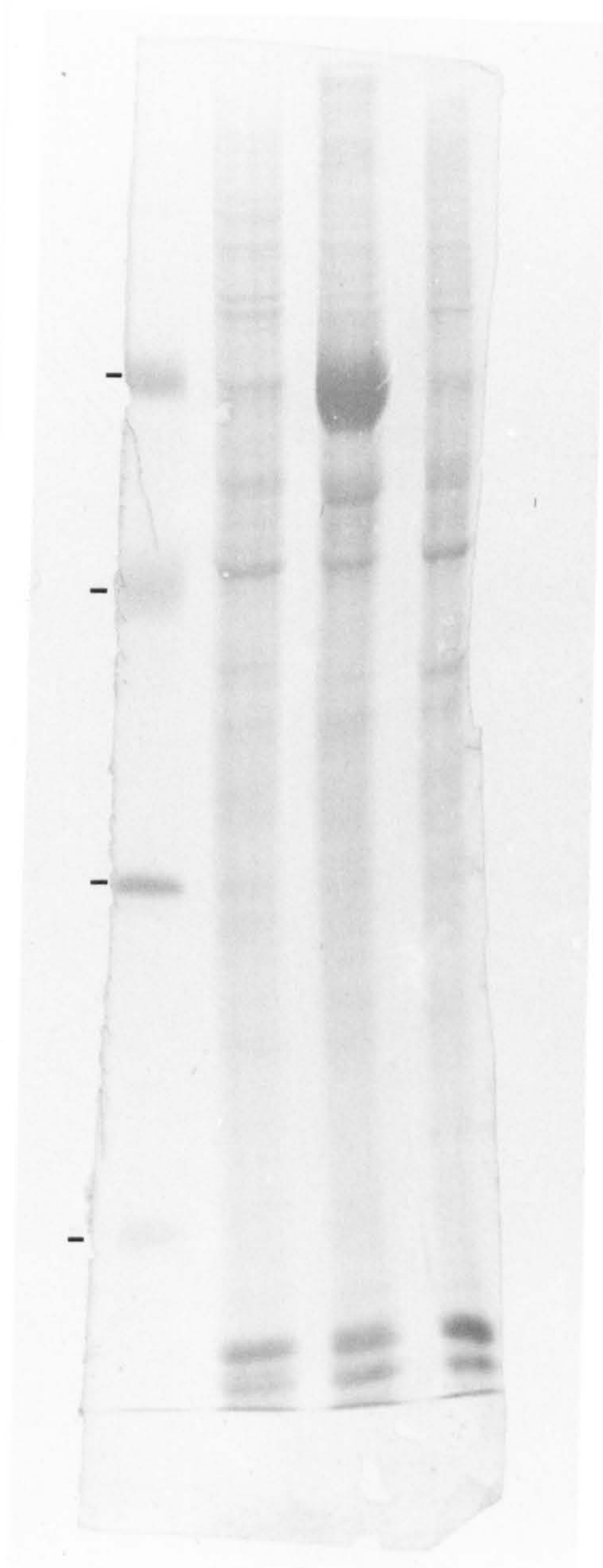


Figure 4.3.15b    Protein Blot of HeLa Subfractions

An amido black stained blot comparing three subcellular HeLa fractions with 2M salt-extracted Chang liver chromatin. Staining and destaining was as described in Fig. 4.3.14b.

- Lane (1)    HeLa cytoplasmic fraction
- Lane (2)    HeLa chromatin
- Lane (3)    HeLa 2M salt-extracted chromatin
- Lane (4)    Molecular weight marker: from top:bovine  
serum albumin, 67,000; ovalbumin, 43,000;  
carbonic anhydrase, 30,000; soybean trypsin  
inhibitor, 20,100
- Lane (5)    Chang liver 2M salt-extracted chromatin

Figure 4.3.15c    Coomassie blue-stained Laemmli gel of HeLa chromatin fractions

Coomassie blue-stained SDS-gel electrophoresis (10%) comparing 2M salt-5M urea extracted-chromatin (lane 1), chromatin preparation (lane 2), 2M salt extracted-chromatin (lane 3) and protein markers (lane 4) [from top: phosphorylase b 94,000, bovine serum albumin 67,000, ovalbumin 43,000, carbonic anhydrase 30,000, soybean trypsin inhibitor 20,100 and  $\alpha$ -lactalbumin 14,400]

Note: In lane 2, although the core histones are well resolved, the H1 histone showed less intensity than the core histones.



Figure 4.3.15b

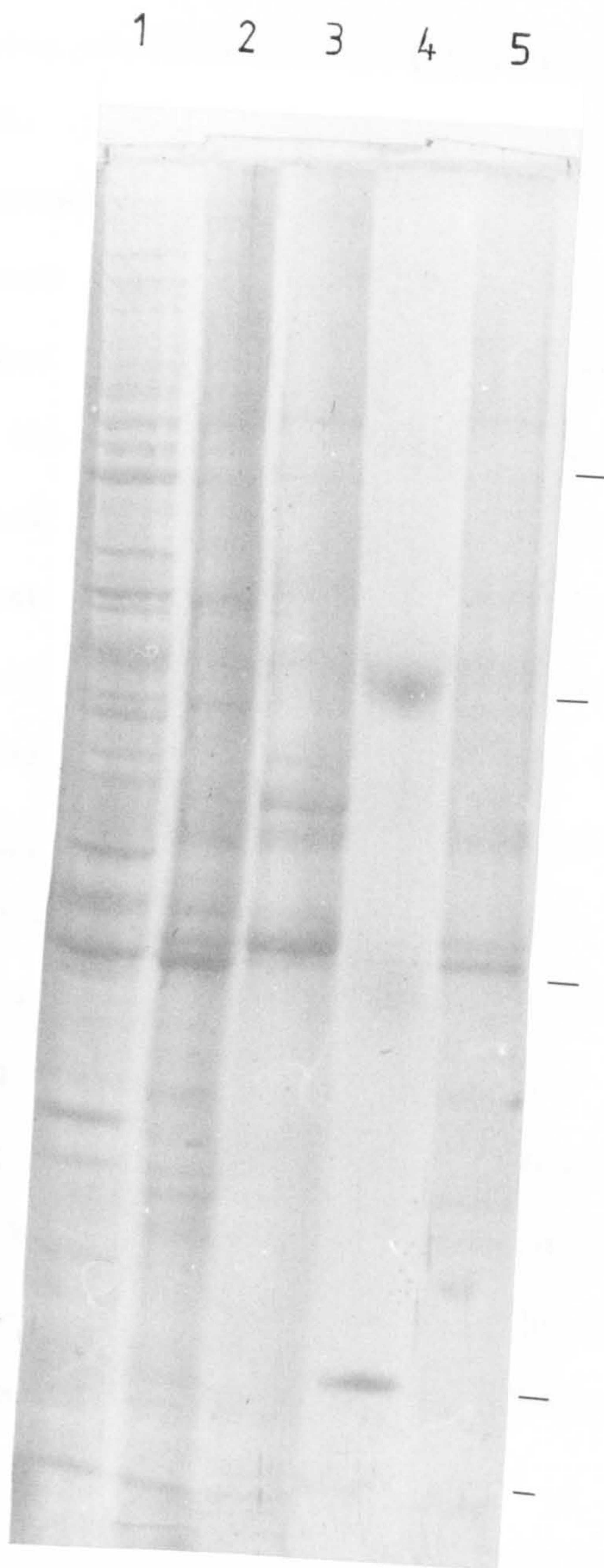
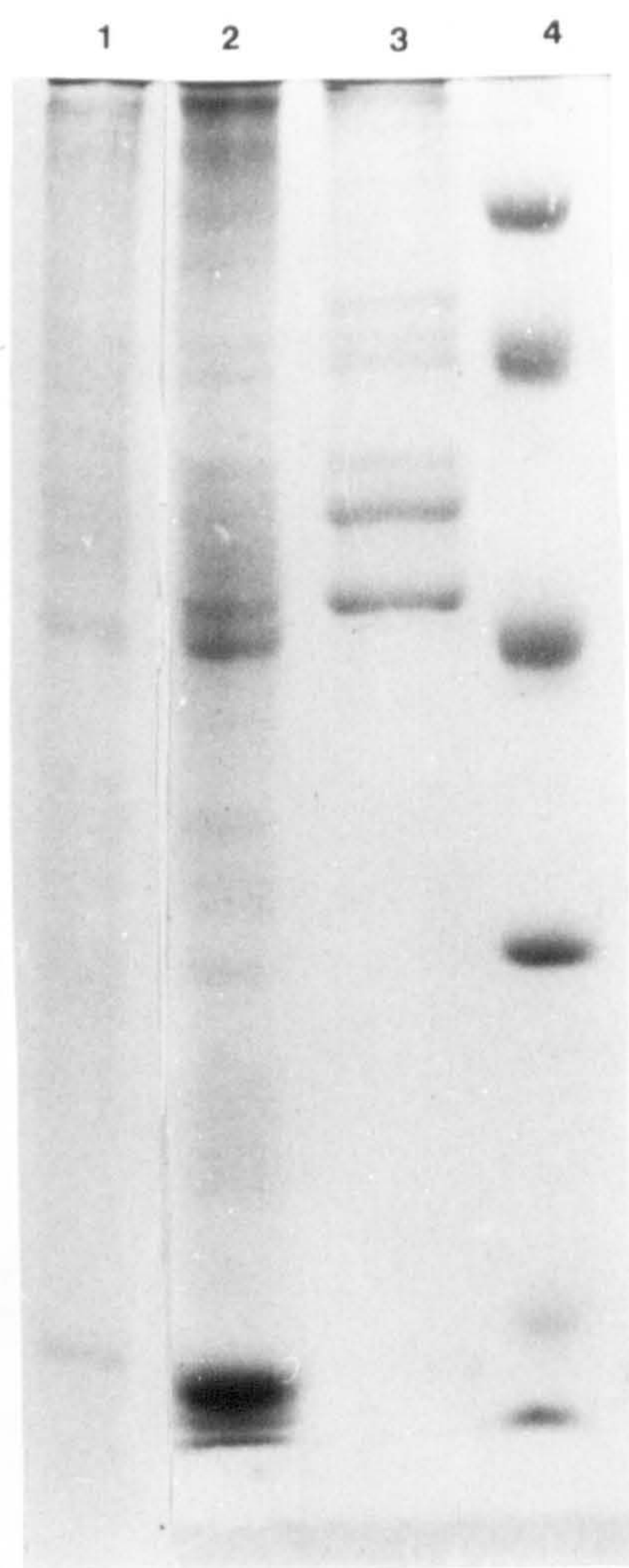


Figure 4.3.15C





either because it is synthesised there or because it performs some cytoplasmic function. Finally, with a monoclonal antibody, the possibility of reaction with the same epitope on two different proteins must always be considered as discussed above.

The data shown in Figure 4.3.10 indicate that the antigen is neither DNA nor RNA. Since antibodies to nucleic acid antigens are not readily raised in BALB/c mice this is not surprising. However, a low level of anti-DNA antibodies is present in the sera of many animals and indeed humans (Winger et al., 1983) and such a possibility must obviously be investigated. The relatively high concentration of DNA (in comparison to any individual protein, see Table 5 for DNA : total protein ratio) in the chromatin used for screening also makes it possible that a low affinity antibody to DNA could have been selected. An antibody to RNA was probably less likely but such monoclonal antibodies have been produced (Eilat et al., 1980, 1982). Antibodies to DNA are readily generated from other strains of mice (Lee et al., 1981; Jacobs & Tron, 1982; Lerner et al., 1981; Kioke et al., 1982) but have not been reported with BALB/c strain.

The fact that DNA and RNA are not required for antigenic activity also indicates that the antigen does not require nucleic acids to maintain its antigenic configuration and in this respect it differs from the HeLa antiserum described by Dunn et al. (1980) which requires human DNA for activity.

Figure 4.3.10 also shows that proteolysis destroys antigenic activity. While the most likely interpretation of this information is that the antigen is a protein, it is also possible that the epitope itself is a carbohydrate residue bound to a protein and removed with the protein fragments on proteolysis.

The immunofluorescence pictures (Fig. 4.3.11) indicate strong nucleolar localisation but also some cytoplasmic presence. While the

most obvious explanation of these data is that the antigen has a presence in both parts of the cell as discussed above, it is also possible that it has changed its location during preparation of the cells for immunohistochemistry. Redistribution of cytoplasmic components to the nucleus has been reported in one case (Briggs et al., 1981) but the nuclear staining showed no specific nucleolar localisation and it seems unlikely that the antigen is a purely cytoplasmic protein.

Since DNA and RNA are not necessary components for the immunoreactivity of HNo-G7 antibody, it is anticipated that the detection of the corresponding antigen on the immunoblot is more feasible. Two nonhistone chromosomal protein preparations were analysed on SDS-PAGE, blotted onto a nitrocellulose paper then allowed to react with the antibody using the immunoperoxidase method (Fig. 4.3.14a lanes c & d). An antigen of approximate molecular weight of 44,500-45,000 was detected. However, the intensity of the reactive band was not strong. This could be explained either by the possibility that a limited amount of antibody was available in the tissue culture supernatant or by the likelihood that the amount of antigen on the blot was not sufficient.

There are many reports in the literature of monoclonal antibodies (particularly to chromatin) which do not immunoblot or which blot poorly (e.g. Turner, 1983). The general interpretation is that such antigens have epitopes which are irreversibly denatured by the preliminary electrophoresis. However, others like Yurchenko et al. (1982) have blotted a variety of protein epitopes successfully. Work in our own laboratory has clearly shown that many sera blot non-specifically with proteins such as molecular weight marker proteins (Campbell et al., personal communication), and the general area of immunoblotting is very much less well defined than indicated in elementary laboratory textbooks. Accordingly, a sandwich blot utilising Protein A was used to identify the antigen more clearly. This blot is based on the known



ability of Protein A to bind strongly to rabbit IgG but not to many mouse monoclonal antibodies and certainly not to mouse IgM monoclonal antibodies. The limited reaction of the mouse specific IgM is amplified with unlabelled rabbit anti-mouse IgM (H+L) antibodies and the final complex is decorated with high specific activity  $^{125}\text{I}$  labelled Protein A. Background staining is reduced by the use of 0.5% Tween 20, which cannot be used successfully with enzyme immunoblots because of the substantial reduction in enzyme activity induced by the bound detergent. This blotting system, which is a modification of the process of Batteiger et al. (1982) has a high signal to noise ratio in comparison to other available techniques. In practice, this technique also indicated that the antigen was a protein in the molecular weight range 45,000 (Fig. 4.3.14b).

The antigenic determinant recognised by HNo-G7 is therefore a protein (possibly with carbohydrate epitopes) which has a molecular weight in the region of 45,000, localised by immunohistochemical techniques primarily in the nucleoli but also present in the cytoplasm, and not dependent on nucleic acids for its immunoreactivity. It is present in smaller amounts or with lower availability in other human epithelial cells but is not apparently available/present in fibrosarcoma cells. A comparison with other published nucleolar antigens is obviously necessary and this is given in Table 6.

The antigens described in Table 6 fall into four separate categories as indicated. The first group of reported "nucleolar specific" antisera are polyclonal (Busch et al., 1979). In view of the potential heterogeneity of such antigens (Section 4.1 and Section 4.2) it is difficult to make a detailed comparison of HNo-G7 with these antisera as they may vary with rabbit, titre and bleed. However, the claims of Busch et al. (1979) that their antiserum specifically detects the nucleoli of all human malignant tumour cells tested indicate that such

Table 6

Nucleolar Antigens

<u>Reference</u>	<u>Molecular weight</u>	<u>Specificity of Antigen</u>	<u>Source</u>
1) Busch <u>et al.</u> (1979) Chan <u>et al.</u> (1980)	54,000	(Human Tumour ( Nucleoli	(Rabbit (Polyclonal
2) Davis <u>et al.</u> (1978) Marachi <u>et al.</u> (1979) Sato & Busch (1983) Lerner <u>et al.</u> (1981)	Various	(Tissue specific (Related to (Metabolic (Activity	(Rabbit (Polyclonal ( & (Autoimmune mouse monoclonal
3) Reddy <u>et al.</u> (1983) Bernstein <u>et al.</u> (1982)	Various	(Limited Tissue (or Species (Specificity	(Human (Polyclonal
4) This work	45,000	(Human Epithelial (Tissue Culture (Cells with Apparent (Preference for HeLa	(Mouse (Monoclonal



antibodies may have possible diagnostic applications. Monoclonal antibody HNo-G7 clearly recognises a different antigenic determinant from such sera since molecular weight determination indicates that their major antigen is substantially larger than that of HNo-G7 (Chan et al., 1980). In addition, the antigen is extracted by low salt (Busch et al., 1979) and this makes it less likely to co-purify with the high salt associated HNo-G7 antigen.

The second group of nucleolar antigens are not necessarily tumour specific but are reported to exhibit some degree of tissue specificity or correlation with growth and metabolic activity (Davis et al., 1978; Marashi et al., 1979; Satoh & Busch, 1983). These have all been detected with polyclonal sera and are subject to the reservations outlined above. The limited characterisation of the antigens in this group makes them difficult to compare with the HNo-G7 antigen but there appears to be little similarity.

The third group of antigens which may be considered as "nucleolar" come from polyclonal human sources, usually from patients with systemic rheumatic diseases involving autoantigens such as progressive systemic sclerosis (Bernstein et al., 1982) and scleroderma (Reddy et al., 1983). Other examples from this group include "cyclin" which may be identical to "proliferating cell nuclear antigen" (Mathews et al., 1984), and others reviewed by Tan (1982). Where molecular weights have been determined, none correlate with the HNo-G7 antigen. Additional complications arise not only from the polyclonality of the serum but also from the autoimmune nature of the response. Such antisera may detect antigens not amenable to conventional immunisation techniques as the host has a specific defect which permits the synthesis of antibodies which are normally suppressed. HNo-G7 was raised in a BALB/c mouse with no autoimmunity in its genetic background.

The final group in Table 6 are genuine monoclonal antibodies which show nucleolar staining. HNo-G7 falls into this category. No other monoclonal antibodies which react with nucleoli have been reported to date. However, a preliminary report of three monoclonal antibodies to purified La antigen is also of interest (Smith et al., 1984). Anti-La antibodies precipitate distinct sets of snRNPs. The group of mouse RNAs associated with these RNPs are of 90-100 nucleotides long (Reviewed by Busch et al., 1982). The structure of human La RNAs is not yet known.

Other monoclonal antibodies to chromosomal proteins have been reported. In particular, two groups have used HeLa cells for immunisation. Bhorjee et al. (1983) reported several monoclonals raised in BALB/c mice with a very similar immunising antigen to the one used to make HNo-G7 (2M salt-5M urea extracted chromatin). However, none of these antibodies showed nucleolar staining. Davis et al. (1983) produced monoclonal antibodies which react strongly with mitotic cells and weakly with interphase cells. Monoclonal antibodies directed to chromatin or chromosomal proteins have also been described utilising Drosophila (Saumweber et al., 1980; Howard et al., 1981), avian red blood cells (Vanderbilt & Anderson, 1982; Kane et al., 1982) and rat and human tissues and cell lines (Turner, 1981) as primary antigen. Many of these antibodies react with antigens that have interesting cell cycle dependence (Howard et al., 1981; Dippold et al., 1981; Risau et al., 1982; Davis et al., 1983) but none show the dramatic nucleolar staining of HNo-G7 which would appear therefore to be a monoclonal antibody recognising a different epitope from others hitherto reported in the literature.



## **General Discussion**

## 5. General Discussion

The class of nonhistone chromosomal proteins remain bound to DNA after extraction with either 2M NaCl alone or with 5M urea has proved exceedingly intractable to biochemical analysis. This is due partly to their complexity and partly to their relative insolubility in the absence of DNA. Their functional role remains therefore obscure but is clearly of general interest. Immunological approaches have considerable potential both in the characterisation of these proteins and the assessment of their distribution within the cell and among different types of cell.

R3.1 antiserum was raised in a rabbit immunised with HeLa dehistonised chromatin extracted with both salt and urea. The major antigens in this serum are in some way structurally dependent on the presence of both protein and DNA (section 4.1). This requirement may reflect simple or complex organisation. For example, protein-protein interaction may be a pre-requisite for DNA binding. Two recent reports of this type of complex reaction support the possibility of this concept. Staufenbiel and Deppert (1984) have shown that a group of chromosomal proteins from HeLa and 3T3 cells interact both among themselves and also with DNA and/or other nuclear structural components. Werner et al. (1984) have also suggested that a form of protein-protein-DNA complex is required for the salt stable anchorage of the residual DNA. In addition, they have reported covalent linkage of some nuclear proteins to DNA. These proteins are highly insoluble when the covalent linkage is broken. It is unlikely that the antigen recognised by R3.1 or reported earlier in HeLa cells (Dunn et al., 1980) is covalently linked since most of the activity can be recovered by reconstitution which would not be expected to result in covalent linkage. However, such a possibility cannot be entirely discounted.



R.3.1 antiserum could obviously be directed against nuclear lamina proteins, nuclear matrix proteins or both. Some of the abundant nuclear matrix and the major lamina proteins lie within the same range of molecular weight but can be differentiated by their structural and functional roles (Agutter & Birchall, 1979; Franke et al., 1981). Staufenbiel and Deppert (1984) have shown that the nuclear lamina is not solubilised during mild DNase digestion or sequential salt extraction of mammalian nuclei in situ. In addition, Lebkowski and Laemmli (1982) have demonstrated that lamina proteins exhibit considerable affinity to bind DNA. On these bases lamina proteins are clear potential candidates for R3.1 major antigens.

The analysis of nuclear matrix proteins by Werner et al. (1984) showed two forms. The minor fraction was that with covalent linkage to DNA referred to above. The major fraction was serologically related to the minor one but not covalently attached to DNA or dependent on DNA for its immunological activity. This pattern does not reflect the behaviour of the major antigens to R3.1.

It is interesting to note that Werner et al. (1984) found nuclear matrix proteins remaining in association with purified eukaryotic DNA. As R3.1 antiserum did not react with purified HeLa DNA, either the preparation of DNA was protein-free or the R3.1 antigens do not represent matrix proteins.

There is, therefore, little evidence to suggest that the major R3.1 antigens are matrix proteins. It is, on the other hand, possible that they represent at least some lamina proteins.

The use of only 2M NaCl and no urea to extract HeLa chromatin results in a different spectrum of major antigens. The nuclear preparation, however highly purified, is generally accompanied by co-sedimenting collapsed intermediate filaments of cytoplasmic origin

(Staufenbiel & Deppert, 1982). This class of protein is resistant to extraction with salt and non-ionic detergents and is likely to contaminate the 2M NaCl-extracted chromatin. The intermediate filaments are highly antigenic (reviewed by Lazarides, 1982). Therefore they may dominate the antiserum produced while forming only a small component of the immunising antigen. As intermediate filament proteins can show considerable cell specificity (Osborn & Weber, 1982), it is very possible that much of the early work which appeared to detect cell specific nuclear proteins was reflecting this type of antigen. The importance of the proof on nuclear localisation of the antigen and establishment of other criteria such as DNA binding is therefore considerable (Briggs et al., 1979; Bowen et al., 1980; Lebkowski & Laemmli, 1982). Since R2.1 antiserum shows a strong filamentous cytoplasmic reaction, it is very likely that this is a result of the presence of antibodies against keratins in the antiserum. This is emphasised by the strong reaction of R2.1 with keratin preparation on the immunoblot at the range of molecular weight covering the intermediate filaments (54,000-61,000 & 42,000-52,000).

The relationship of the major antigens in R2.1 to nuclear lamina or matrix proteins is not clear. The immunoblotting of R2.1 antiserum shows no indication of the major lamina proteins of molecular weight 68,000-70,000 although the nuclear staining shows a similar pattern to that of an anti-lamina monoclonal antibody recently reported by Krohne et al. (1984). The nuclear matrix is relatively stable under the conditions of antigen extraction, however, and could have been present in the immunising antigen (Capco et al., 1982). The matrix network in HeLa cells appear to remain connected to the cytoskeleton after extraction with high salt buffer and can be shown by electron microscopy to be more dense than the cytoskeleton (Capco et al., 1982). In addition, lamina



proteins have also been reported to be stable under similar conditions (Lebkowski & Laemmli, 1982). The relatively high density of FITC-staining surrounding the nuclei may therefore possibly represent antigens present on the nuclear envelope or matrix.

Capco et al. (1982) also showed that extraction of nuclei at high ionic strength buffer yielded a filamentous nuclear network well organised in a three-dimensional anastomosing structure where the nucleoli are enmeshed. The intranucleolar filaments are situated deep within the nucleus. In situ fractionation has shown the persistence of preserved nucleolar structures during high salt extraction (Staufenbiel & Deppert, 1984). Consequently, the presence of nucleolar components in chromatin which has been extracted with 2M NaCl 5M urea is not surprising and the HNo-G7 antibody presumably detects such a component. It is interesting to note that the R3.1 antiserum shows no comparable antigen. The difference could be due to the variation in immunological response between rabbit and mouse but is more likely to reflect the ability of monoclonal antibody technology to select antibodies directed to a specific protein which may be a minor serum antigen.

The main structural components of the nucleolus are thought to be condensed ribosomal DNA, histones, RNA species and ribosomal protein precursors. The HNo-G7 antigen is a protein which has no obvious association with RNA or DNA (section 4.3). The data suggest that it is not a histone. It remains possible that it is a ribosomal protein or a precursor of such a protein. Alternatively, it may be a protein which plays a role in nucleolar structure or function but bears no serological relationship to ribosomal proteins.

The molecular weight of the HNo-G7 antigen is close to that of actin. In this context it is interesting to note the prevalence of actin in preparations of chromatin and nuclear matrix proteins (Capco et

al., 1982; Bravo et al., 1982; LeStourgeon et al., 1975). It seems unlikely, however, that HNo-G7 is reacting with actin since the immunofluorescence is not characteristic of anti-actin antibodies described by Weber et al. (1976). Another major protein which has recently been shown to co-focus with actin (using two-dimensional gel electrophoresis) in HeLa cytoskeletal, chromatin and matrix preparations (Capco et al., 1982) is of interest. The protein is resistant to the extraction conditions used in these preparations and its molecular weight is similar to actin. However, it is not clear, from this report (Capco et al., 1982), whether this protein is of nucleolar localisation or not.

The work described in this thesis has emphasised the complexity not only of nuclear proteins but also of the contaminants which can co-purify with them. Immunological analysis is of particular value in such a situation, since nuclear localisation or the lack of it can be clearly established by immunocytochemical techniques. Such antigens can then be further investigated and purified by immunological means. The comparative simplicity of the use of a monoclonal antibody as opposed to conventional sera to identify nuclear antigens is also evident.

A striking feature of current advances in recombinant DNA technology is that even when the entire sequence of a eukaryote gene has been established, in the majority of cases little can be deduced about the control of its differential expression in the cell. It is to be hoped that immunological analysis of the chromosome may help in the identification of some of the molecules involved in this task.



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